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Molecular Networking to Screen Macroalgal Secondary Metabolites: Case for West Timor Macroalgae

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Abstract

The research describes how molecular networking was used as a screening tool to prioritize the isolation workflow of 40 macroalgae sampled from West Timor waters, Indonesia, in addition to a Nuclear Magnetic Resonance-based (NMR) spectroscopy strategy. A mass spectrometry (MS) was employed to generate spectra that later be used as data to produce the molecular network with the Global Natural Product Social Molecular Networking (GNPS) website. The screening process used the molecular network, which assisted in the selection of six samples of macroalgae out of the 40 samples for further examination. Lastly, an NMR-based protocol was employed to choose the samples of interest to be investigated further. Six samples were shortlisted from the GNPS molecular network based on specimens, which were then validated with ^{1}H NMR spectroscopy to finally prioritized three samples.

Keywords: GNPS, Macroalgae, ^{1}H NMR, Molecular networking, Spectra

INTRODUCTION

After an organism has been chosen as a species of interest, the next step is preliminary solvent extraction which results in crude extracts. Once the crude extracts are obtained, various screening methods are employed to highlight those worthy or more in-depth investigations. The traditional approach has been bioassay-guided screening, which usually leads to isolating biologically active compounds (Eloff, 1998; Hostettmann, Wolfender, & Terreaux, 2001; Koehn & Carter, 2005). Although the bioassay-guided screening eventually produces a potential pharmaceutical compound, the frequent re-isolation of previously known metabolites is a significant challenge for this strategy (Koehn, 2008; Koehn & Carter, 2005).

Alternative approaches have been introduced as screening tools, namely spectroscopy-guided screening utilizing NMR spectroscopy or MS coupled with liquid chromatography (LC-MS). This newer strategy, combined with high-quality spectral databases, can help identify known compounds in the extracts or active substances that have already been studied (Ito & Masubuchi, 2014; Koehn, 2008). The procedure is known as de-replication, a term first coined in the CRC Handbook of Antibiotic Compounds published in 1980 (Ito & Masubuchi, 2014). Dereplication provides rapid identification of known compounds within crude extracts or semi-purified mixtures, facilitating prioritization for further elucidation of only potentially new structures and halting an isolation process of known secondary metabolites (Ito & Masubuchi, 2014; Moser, Wheeler, & Hayward). Hence, de-replication is a substantial and vital strategy in natural product screening.

Secondary metabolites are typically amphiphilic, which allows the compounds to transverse both hydrophilic and hydrophobic environments. Their vast polarity and solubility make it challenging to analyze and handle the compounds present in the initial purification of a crude extract. In particular, finding single solvents that will dissolve all the components of a mixture is difficult, if not impossible, without some level of pre-fractionation. Preliminary purification steps have commonly been done via liquid/liquid partitioning. However, as some extracts may form stable emulsions (John W Blunt et al., 1987), leading to the incomplete partitioning of the extract and target compounds and the requirement of large volumes of solvent, this

straightforward technique has been changed to column chromatography. The widely used reversed-phase chromatography procedure developed by Blunt and Munro has been used for many years to overcome problems associated with standard flash column chromatography (normal phase) (John W Blunt et al., 1987). In living systems, most organisms have molecules that are either very non-polar (lipids/steroids) or very polar (proteins/sugars). However, most interesting (amphiphilic) natural products are frequently not discovered with these molecules but in the intermediate "mass window" (Woolner, 2017).

At Victoria University of Wellington (VUW), the reversed-phase polystyrene divinylbenzene (PSDVB) copolymer (HP20) was found to have the advantage of being a relatively inexpensive adsorbent, stable throughout the pH range, and reusable, unlike silica gel (normal phase). Also, acetone and methanol, environmentally friendly solvents, are widely used for this technique, giving an additional advantage. This method is known as cyclic loading and has benefited the natural product research group at VUW for quite some time. The cyclic loading system enables crude extracts to be loaded directly onto the PSDVB column without pre-concentration. Popplewell (Popplewell, 2008), who previously studied 34 temperate New Zealand red algae in the marine natural products group at VUW, developed a method for algal screening modified from VUW in-house protocols designed for screening sponge extracts (Keyzers, 2003; Popplewell, 2008). The protocol involves the extraction from 2 g or more (wet weight) algal material because macroalgae have a more affluent fraction of secondary metabolites than sponge extracts which typically require ~ 100 g wet weight (Popplewell, 2008). Since the mid-1990s, the MNPs group at VUW has been utilizing NMR-guided isolation of secondary metabolites.

Mass spectrometry (MS) has become a standard procedure for investigating complex mixtures and molecules. Liquid chromatography coupled with mass spectrometry (LC-MS) is a hyphenated analytical technique that synergizes the ability to perform fractionation via liquid chromatography with the mass analysis capability of MS (Korfmacher, 2005). This technique works by ionizing a molecule that is smashed and turned into charged fragments, which then would be quantitated based on their mass-to-charge (m/z) ratio (Bythell, Hendrickson, & Marshall, 2012; Vaiano et al., 2015). An LC-MS system includes elements such as high-performance liquid chromatography (HPLC) system; the ionization source (which interfaces the LC to the MS); and the mass spectrometer. A computer system is used to control all these elements (Korfmacher, 2005). The HPLC system prepares a mixture of water and common hydrophilic solvents (isopropanol, methanol, or acetonitrile) as the mobile phase. A pumping mechanism in the HPLC system allows pressurized liquid and a sample mixture to pass through a column filled with the adsorbent stationary phase. The adsorbent is typically a granular material (1–50 μm) made of solid particles (typically a silica-based bonded phase, e.g., C18) (Niessen, 2003). The ionization source is used as the interface between the HPLC eluent and the mass spectrometer. Two standard atmospheric-pressure ionization (API) technique sources are electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI). The eluent from the LC is nebulized to produce ions from the evaporating droplets. Nebulization is achieved either pneumatically via APCI or by a strong electrical field in ESI (Niessen, 2003). Several mass analyzers are available, but nowadays, time-of-flight (TOF) MS is the most widely used system in drug discovery research. In a quadrupole time-of-flight (QTOF) instrument, as used in this study, the quadrupole is used to select precursor ions that will be fragmented later in a collision cell. These generated ions are then separated by the TOF and detected by a photo-electron multiplier plate (Loboda, 2014).

METHODOLOGY

Although a single LC-MS experiment is a powerful technique that can collect thousands of spectra relatively briefly, most data is only sitting in the researchers' drawers or computers. Moreover, most natural products databases such as the Dictionary of Natural Products (http://dnp.chemnetbase.com/) and MarinLit (http://pubs.rsc.org/marinlit) only provide services to their subscribers. Recognizing this need, the University of California San Diego (UCSD) Centre for Computational Mass Spectrometry (http://proteomics.ucsd.edu) developed Global Natural Product Social Molecular Networking (GNPS) to accommodate the demand for robust dereplication of natural products that are freely

available to the global research community (Wang et al., 2016). GNPS is an open database that can analyze, organize, and create networks from tandem mass spectra data (Wang et al., 2016). Moreover, the publicly available GNPS database (known as MassIVE) is used to compare experimental data with the known spectra library, which is helpful for dereplication in natural products.

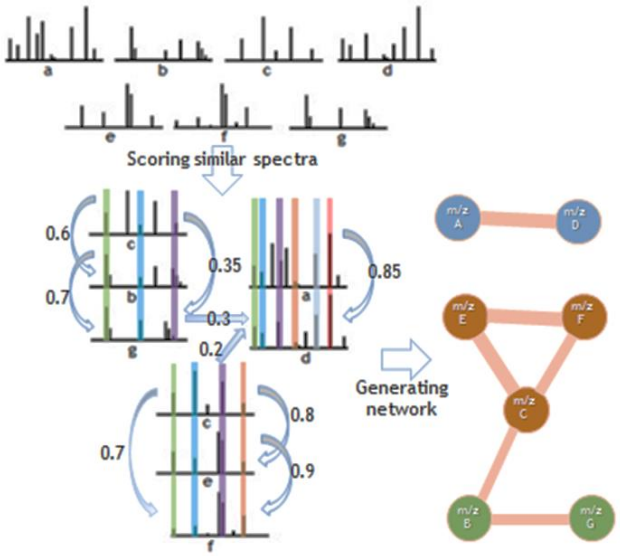

Figure 1. A scheme shows how molecular networks are created from LC-MS data (Modified from Watrous (Watrous et al., 2012) used by permission)

Instead of a linear comparison, GNPS uses a vector-based approach to match two or more different MS/MS spectra (see Figure 1 for details). Experimental mass spectra with unique fragmentation patterns are combined into multidimensional vectors. Vectorization in GNPS happens by taking not only the peak intensity but also the ion's mass-to-charge ratio (m/z). An overall vector is generated by plotting the m/z ratio of multiple peaks (*n*) in *n*-dimensional space. Each overall vector represents a compound or potentially isomers and will be shown as nodes. The overall vectors of different compounds can be aligned and compared with each other; thus, the cosine of the angle between two or more vectors can be used to measure their similarity. The cosine score represents how closely related two nodes (hence compounds) are, which varies from 0 (completely unrelated) to 1 (identical spectra). In the network, the cosine score is usually represented by the thickness of a line (edge) that connects two nodes.

A cluster is formed when edges connect nodes and comprise a unique set of related molecules as structurally similar compounds tend to have similar properties and belong to the same group (Morrow, Tian, & Zhang, 2010; Nguyen et al., 2013). GNPS also enables the annotation of putative nodes; therefore, the mass difference can be used to annotate other nodes (Sumner et al., 2007). GNPS provides network visualization on their website (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash2.jsp). However, a more sophisticated third-party software, called Cytoscape, is available to visualize the network (http://www.cytoscape.org) (Shannon et al., 2003; Smoot, Ono, Ruscheinski, Wang, & Ideker, 2010). This open-source freeware visualizes critical defining attribute values in the network in different shapes, colors, and sizes (Cline et al., 2007). Cytoscape is a reliable tool for displaying large data sets in other areas such as metabolomics, biochemical pathways, population networks, and even social science research (Boya P et al., 2017; Kofia, Isserlin, Buchan, & Bader, 2015; Watrous et al., 2012; Zhou, Shaverdian, Jagadish, & Michailidis, 2009). The current freeware version is Cytoscape v3.9.1 (released in January 2022).

The research was carried out in Timor Island and Semau Island coastal waters, East Nusa Tenggara Province, Indonesia. Four locations were visited to collect fresh macroalgae samples (Figure 2). Three of the sites are in Timor Island i.e., Sulamu Beach (10° 3' 5.463" S and 123° 36' 52.29" E), Pasir Panjang Beach (10° 8' 55.464" S and 123° 36' 13.356" E), and Tablolong Beach (10° 19' 2.136" S and 123° 28' 13.728" E). While another collection site, Akle Beach, is on Semau Island (10° 19' 20.7048" S and 123° 20' 12.444" E). Fresh macroalgae were sampled in the intertidal area during the low tide period. Samples were collected in water no deeper than knee height. A total of 40 samples of different species of brown, red, and green macroalgae were collected from all four sites (Table 1). The number of green algae (Chlorophyta) and red alga (Rhodophyta) collected were the same, while fewer brown alga (Phaeophyta) was sampled. Interestingly, Akle Beach in Semau Island provided more species than the other three beaches.

Prior to extraction, samples were dried under the sun for two to three days, labelled and stored. The extraction was done in the Integrated Science Laboratory of Universitas Nusa Cendana, Kupang,

Indonesia. The samples were macerated and extracted twice in methanol for 24-hour periods. Both extracts were then combined. The methanol extract was subsequently concentrated in vacuo to dry samples completely or remove the methanol. As the last step, the remaining solution (presumably water) was removed in a freeze dryer at -100^{o}C to -121^{o}C. The dried samples were finally obtained and sent to Victoria University of Wellington for further analysis.

Table 1. Number of macroalgal collected on each site based on phylum

Location	Number of Phaeophyta (brown)	Number of Rhodophyta (red)	Number of Chlorophyta (green)	Total
Sulamu Beach	4	3	2	9
Pasir Panjang Beach	-	4	2	6
Akle Beach	4	6	10	20
Tablolong Beach	2	2	1	5
Total	10	15	15	40

Figure 2. Sampling locations of macroalgae from West Timor waters, Indonesia. The map retrieved from https://www.google.com/maps/@-10.1345414,123.5736125,70500m/data=!3m1!1e3 (used by permission)

RESULTS AND DISCUSSION

The 40 macroalgae samples collected from West Timor waters in Indonesia were extracted using methanol (MeOH) at room temperature and were dried afterward to obtain a crude extract weight. Before LC-MS analysis, all samples were diluted in MeOH to a set of concentrations (0.1 µg µL^{-1}). In the LC-MS, both positive and negative modes were employed to get fragments of the samples based on their m/z ratio to maximize the amount of data obtained since some compounds only ionized under one mode. Spectra data from LC-MS/MS were converted and exported as mgf files to the GNPS website along with text files of meta-data attributes to produce the network. On the GNPS website, each positive or negative ion mode dataset was run separately. All essential parameters were set to create consensus spectra: parent mass tolerance was set to 0.02 Da, and MS/MS fragment ion tolerance was set at 0.02 Da. Also, the consens5us spectra that contained less than two spectra were discarded. The Minimum Pairs Cosines score was set to 0.7, and the minimum fragment ions matched was set to six data to produce the network. The results were then exported and later visualized in the Cytoscape application (version 3.7) and displayed in “preferred layout” settings. Positive mode data showed 574 nodes and 857 edges (Figure 3), while negative mode showed fewer nodes and edges, 120 and 182, respectively (Figure 4).

The positive ion GNPS network shows that macroalgae collected from Akle Beach have the potential for further study (Figure 3). The network is dominated by clusters formed from algae collected at Akle Beach (more than 10 clusters), followed by two clusters from Sulamu Beach and Tablolong Beach (which connect to a Sulamu Beach cluster), with no clusters from Pasir Panjang Beach. Akle Beach has the most clusters since 20 out of 40 macroalgae samples were collected from this site. Conversely, in the negative ion mode, no single cluster was formed from algae collected from a specific location as they have nodes that also belong to extracts from other sites (Figure 4).

However, the negative ion network also confirms that potential macroalgae for screening come from similar beaches, as shown in the positive ion mode (i.e., Akle Beach, Sulamu Beach, and Tablolong Beach). This may occur due to the production of secondary metabolites from macroalgae as chemical defenses against herbivores. As Pasir Panjang Beach is located in urban areas, the absence of herbivory fish in this site might slightly alter the function of secondary metabolites on this particular beach (Pereira & Da Gama, 2008; Schmitt, Hay, & Lindquist, 1995). When the phylum of macroalgae is considered for grouping the GNPS network (Figures 5 and 6), green macroalgae

(Chlorophyta) were shown as the most prolific source of compounds, followed by brown (Phaeophyta) and red macroalgae (Rhodophyta). Thirteen clusters were formed from green algae, while brown and red algae generated nine and seven clusters, respectively. These results contradict the trend in macroalgae secondary metabolites research since the most prolific source of macroalga natural products is red algae which account for more than 50%, followed by brown for almost 40%, and the rest are from green seaweed (Hasanela, N., & Souhoka, F. A., 2022; Telussa, I., Hattu, N., & Sahalessy, A., 2022; Bijang, C. M., Tehubijuluw, H., & Kaihatu, T. G., 2018; John W Blunt, Copp, Keyzers, Munro, & Prinsep, 2014; John W Blunt, Copp, Keyzers, Munro, & Prinsep, 2013, 2015, 2016; John W. Blunt, Copp, Keyzers, Munro, & Prinsep, 2017; Cabrita, Vale, & Rauter, 2010; Leal et al., 2013).

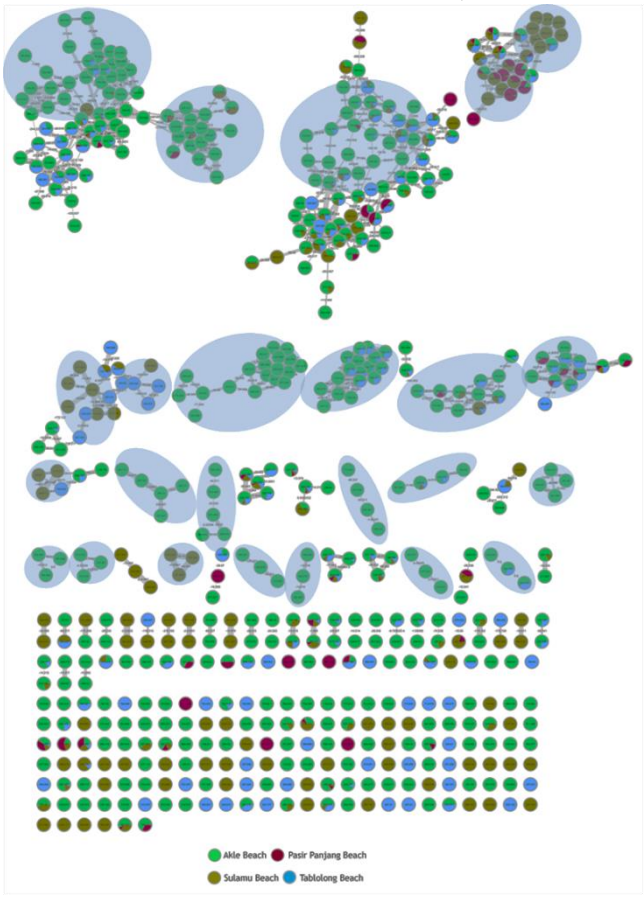

Figure 3. GNPS network of macroalgae based upon sampling locations in positive ion mode. Colors are arbitrarily assigned to collection site

However, the seasonal assemblage of intertidal macroalgae in the tropics usually shifts to more green and red algae in late summer/the rainy season (mainly between Oct-Dec each year)(Ormond & Banaimoon, 1994) The samples collected from West Timor waters found more green and red macroalgal species than brown during the Oct-Nov sampling. This result suggested brown algae may be an understudied resource for finding new marine natural products.

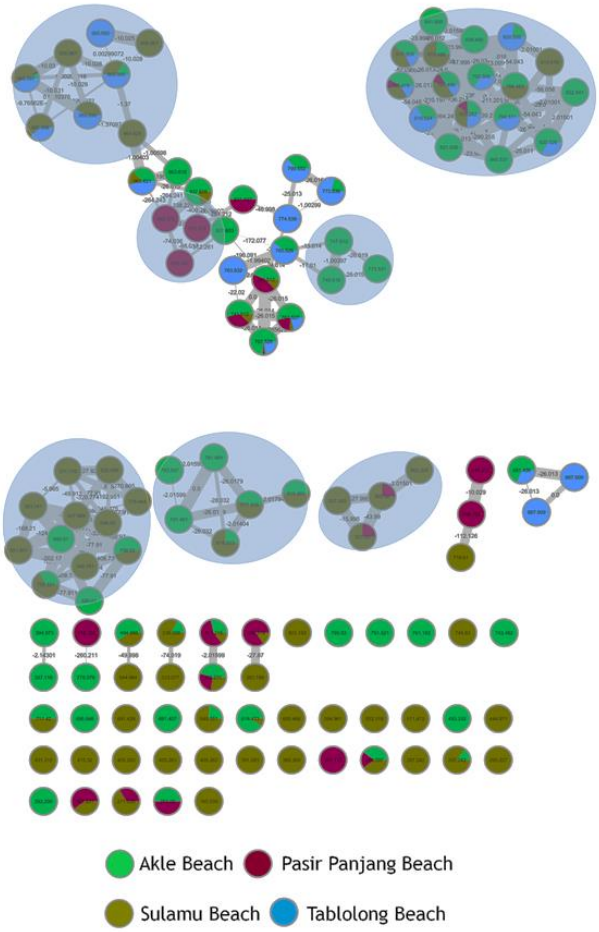

Figure 4. GNPS network of macroalgae based upon sampling locations in negative ion mode. Colors are arbitrarily assigned to collection site

Molecular networking based on individual species enabled a selection of samples to be prioritized in the isolation workflow. Six samples were selected from both positive and negative mode networks to be processed further for NMR screening (Figure 7). The samples are *Amphiroa* sp1 (WEM_01_005) from Sulamu Beach; *Amphiroa* sp2 (WEM_03_002), *Ulva* sp1 (WEM_03_004), and *Ulva* sp2 (WEM_03_005) from Pasir Panjang Beach; *Padina* sp2 (WEM_04_019) from Semau Island, *Laurencia snackeyi* (WEM_05_001) from Tablolong Beach (Figure 9). These samples were selected based on clusters formed where the clusters were dominated by one of the six specimens (Figure 7).

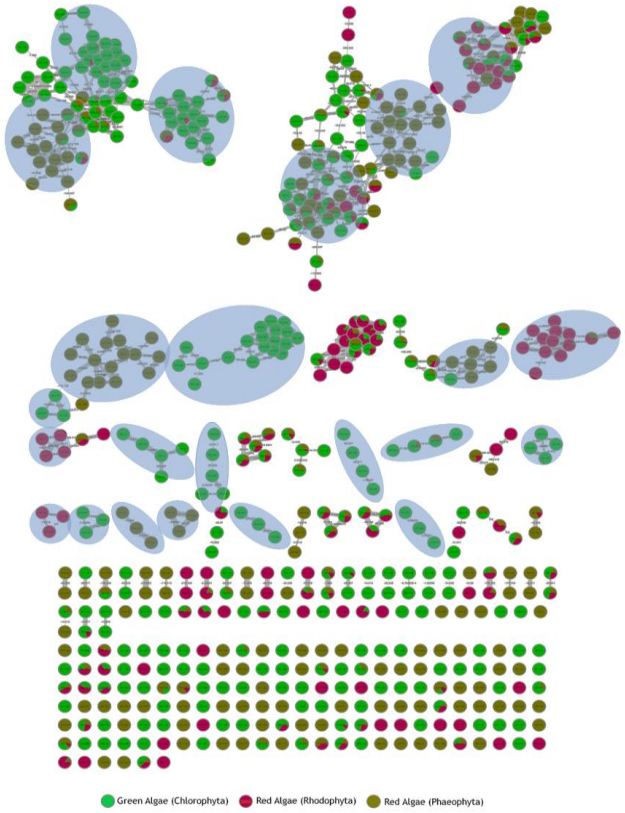

Figure 5. GNPS network of macroalgae based upon phylum in positive ion mode. Colors are arbitrarily assigned to collection site

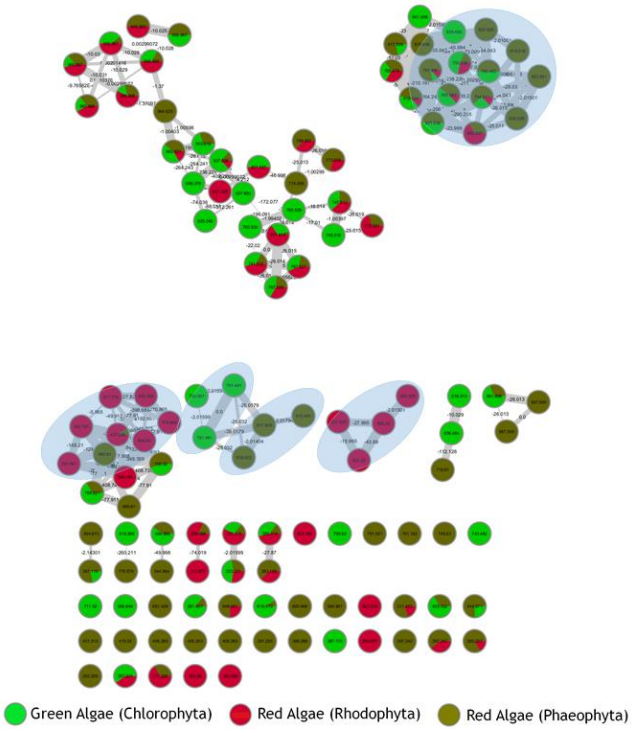

Figure 6. GNPS network of macroalgae based upon phylum in negative ion mode. Colors are arbitrarily assigned to collection site

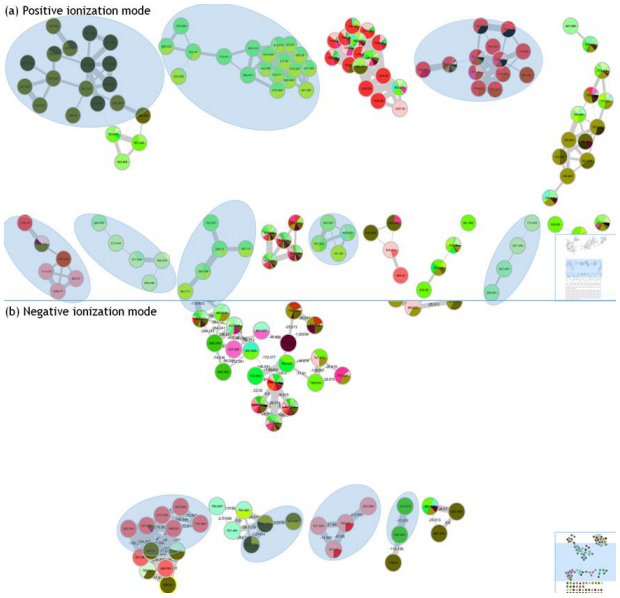

Figure 7. GNPS network of macroalgae based on specimens. Colors are arbitrarily assigned to 40 different samples of species

Six extracts that had been prioritized through molecular networking (previous section), were then fractionated before using ^{1}H NMR to verify the presence of interesting secondary metabolites. Although NMR spectroscopy does not offer biological information, it is perceived that novel structures often lead to interesting biological activity (Keyzers, **2003**). As argued before, the intermediate 75% fraction (mass window) showed the most interesting peaks compared to the 30% and 100% Me_2CO fractions.

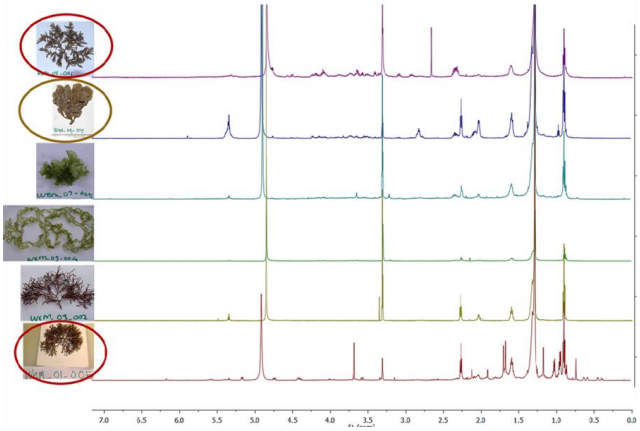

Figure 8. ^{1}H NMR spectra from 75% semi-purified fractions of six prioritized samples of macroalgae (600 MHz, CD_3OD)

Out of six samples, three were initially chosen as having interesting peaks between 3.5-5 ppm (Figure 8); namely WEM_01_005 (*Amphiroa* sp1), WEM_04_019 (*Padina* sp2), and WEM_05_001 (*Laurencia snackeyi*). The resonances in these downfield correspond to oxymethine protons, predominantly sugars signals (Figure 8).

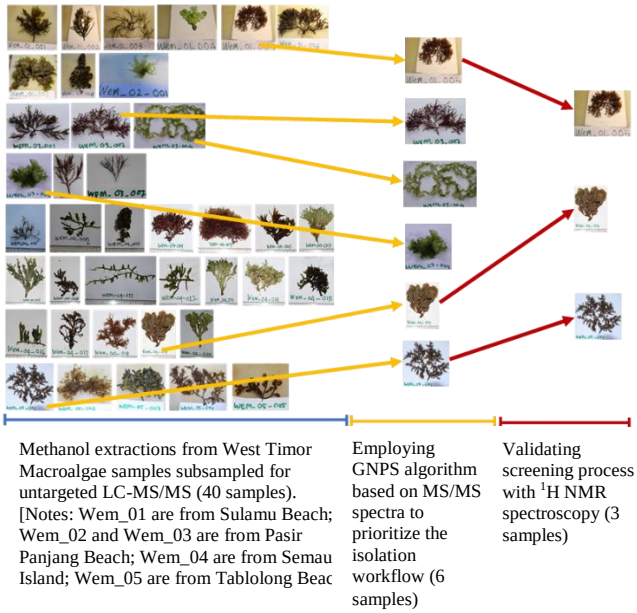

Figure 9. Prioritization process that showing how dereplication by GNPS has helped in prioritizing isolation workflow

CONCLUSION

This study began with the sampling of 40 specimens of tropical Indonesian macroalgae from West Timor waters. A molecular networking screening was employed to priorities the isolation workflow. The crude extracts of the samples were analyzed using liquid chromatography-mass spectrometry (LC-MS)/mass spectrometry (MS) to generate data for creating the molecular network through the Global Natural Product Social Molecular Networking (GNPS) platform. Based on the clusters formed, six samples were prioritized from the molecular network. The suggestions from the screening process were then validated with ^{1}H NMR spectroscopy, which finally prioritised three samples.

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REFERENCES

Bijang, C. M., Tehubijuluw, H., & Kaihatu, T. G. (2018). Biosorpsi Ion Logam Kadmium (Cd^{2+}) Pada Biosorben Rumput Laut Coklat (Padina australis) Asal Pantai Liti Pulau Kisar. *Indo. J. Chem. Res.*, *6*(1), 51-58.

Blunt, J. W., Calder, V. L., Fenwick, G. D., Lake, R. J., McCombs, J. D., Munro, M. H., & Perry, N. B. (1987). Reverse Phase Flash Chromatography: A Method for The Rapid Partitioning of Natural Product Extracts. *Journal of Natural Products*, *50*(2), 290-292.

Blunt, J. W., Copp, B. R., Keyzers, R. A., Munro, M. H., & Prinsep, M. R. (2013). Marine Natural Products. *Nat. Prod. Rep.*, *30*(2), 237-323.

Blunt, J. W., Copp, B. R., Keyzers, R. A., Munro, M. H., & Prinsep, M. R. (2015). Marine Natural Products. *Nat. Prod. Rep.*, *32*(2), 116-211.

Blunt, J. W., Copp, B. R., Keyzers, R. A., Munro, M. H., & Prinsep, M. R. (2016). Marine Natural Products. *Nat. Prod. Rep.*, 33, 382-431

Blunt, J. W., Copp, B. R., Keyzers, R. A., Munro, M. H. G., & Prinsep, M. R. (2017). Marine Natural Products. *Nat. Prod. Rep.*, *34*(3), 235-294.

Boya P, C. A., Fernández-Marín, H., Mejía, L. C., Spadafora, C., Dorrestein, P. C., & Gutiérrez, M. (2017). Imaging Mass Spectrometry and MS/MS Molecular Networking Reveals Chemical Interactions Among Cuticular Bacteria and Pathogenic Fungi Associated with Fungus-Growing Ants. *Scientific Reports*, *7*(1), 5604.

Bythell, B. J., Hendrickson, C. L., & Marshall, A. G. (2012). Relative Stability of Peptide Sequence Ions Generated by Tandem Mass Spectrometry. *Journal of The American Society for Mass Spectrometry*, *23*(4), 644-654.

Cabrita, M. T., Vale, C., & Rauter, A. P. (2010). Halogenated Compounds from Marine Algae. *Marine drugs*, *8*(8), 2301-2317.

Cline, M. S., Smoot, M., Cerami, E., Kuchinsky, A., Landys, N., Workman, C., . . . Gross, B. (2007). Integration of Biological Networks and Gene Expression Data Using Cytoscape. *Nature protocols*, *2*(10), 2366.

Eloff, J. N. (1998). A Sensitive and Quick Microplate Method to Determine The Minimal Inhibitory Concentration of Plant Extracts for Bacteria. *Planta Med*, *64*(8), 711-713.

Hasanela, N., & Souhoka, F. A. (2022). Effect of Heating Coarse Extract of Brown Macroalgae (Padina australis) from Tial Waters, Salahutu District, Central Maluku Regency on Antioxidant Activity. *Indo. J. Chem. Res.*, 10(2), 102-109.

Hostettmann, K., Wolfender, J.-L., & Terreaux, C. (2001). Modern Screening Techniques for Plant Extracts. *Pharmaceutical biology*, *39*(sup1), 18-32.

Ito, T., & Masubuchi, M. (2014). Dereplication of Microbial Extracts and Related Analytical Technologies. *The Journal of antibiotics*, *67*(5), 353.

Keyzers, R. A. (2003). The Isolation of Biologically Active Secondary Metabolites From New Zealand Marine Organisms. *Dissertation*, Victoria University of Wellington,

Koehn, F. E. (2008). High impact Technologies For Natural Products Screening. In *Natural Compounds as Drugs Volume I* (pp. 175-210): Springer.

Koehn, F. E., & Carter, G. T. (2005). The Evolving Role of Natural Products in Drug Discovery. *Nature Reviews Drug Discovery*, *4*(3), 206-220.

Kofia, V., Isserlin, R., Buchan, A. M. J., & Bader, G. D. (2015). Social Network: a Cytoscape app for visualizing co-authorship networks. *F1000Research*, *4*, 481-481.

Korfmacher, W. A. (2005). Foundation Review: Principles and Applications for LC-MS in New Drug Discovery. *Drug discovery today*, *10*(20), 1357-1367.

Leal, M. C., Munro, M. H., Blunt, J. W., Puga, J., Jesus, B., Calado, R., . . . Madeira, C. (2013). Biogeography and Biodiscovery Hotspots of Macroalgal Marine Natural Products. *Natural Product Reports*, *30*(11), 1380-1390.

Loboda, A. (2014). *Method and System for Operating A Time of Flight Mass Spectrometer Detection System*. In: Google Patents.

Morrow, J. K., Tian, L., & Zhang, S. (2010). Molecular Networks in Drug Discovery. *Critical Reviews™ in Biomedical Engineering, 38*(2), 143-156

Moser, A., Wheeler, P., & Hayward, S. (2005). Dereplication of Natural Products by NMR: A Three-Stage Approach ACD/Structure Elucidator Suite and ACD/Labs' Content Databases.

Nguyen, D. D., Wu, C.-H., Moree, W. J., Lamsa, A., Medema, M. H., Zhao, X., . . . Jackson, C. (2013). MS/MS Networking Guided Analysis of Molecule and Gene Cluster Families. *Proceedings of the National Academy of Sciences*, *110*(28), E2611-E2620.

Niessen, W. (2003). Progress in Liquid Chromatography–Mass Spectrometry Instrumentation and Its Impact on High-Throughput Screening. *Journal of chromatography A*, *1000*(1), 413-436.

Ormond, R., & Banaimoon, S. (1994). Ecology of Intertidal Macroalgal Assemblages on the Hadramout Coast Of Southern Yemen, an Area of Seasonal Upwelling. *Marine Ecology-Progress Series*, *105*, 105-105.

Pereira, R. C., & Da Gama, B. (2008). Macroalgal Chemical Defenses and Their Roles in Structuring Tropical Marine Communities. In *Algal chemical ecology* (pp. 25-55): Springer.

Popplewell, W. L. (2008). Isolation and Structure Elucidation of New Secondary Metabolites from New Zealand Marine Red Algae.*Dissertation*, Victoria University of Wellington,

Schmitt, T. M., Hay, M. E., & Lindquist, N. (1995). Constraints on Chemically Mediated Coevolution: Multiple Functions for Seaweed Secondary Metabolites. *Ecology*, *76*(1), 107-123.

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., . . . Ideker, T. (2003). Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome research*, *13*(11), 2498-2504.

Smoot, M. E., Ono, K., Ruscheinski, J., Wang, P.-L., & Ideker, T. (2010). Cytoscape 2.8: New Features for Data Integration and Network Visualization. *Bioinformatics*, *27*(3), 431-432.

Sumner, L. W., Amberg, A., Barrett, D., Beale, M. H., Beger, R., Daykin, C. A., . . . Viant, M. R. (2007). Proposed Minimum Reporting Standards for Chemical Analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics: Official journal of the Metabolomic Society*, *3*(3), 211-221.

Telussa, I., Hattu, N., & Sahalessy, A. (2022). Morphological Observation, Identification and Isolation of Tropical Marine Microalgae from Ambon Bay, Maluku. *Indo. J. Chem. Res*., 9(3), 137-143.

Vaiano, F., Serpelloni, G., Focardi, M., Fioravanti, A., Mari, F., & Bertol, E. (2015). LC–MS/MS and GC–MS Methods in Propofol Detection:

Evaluation of the Two Analytical Procedures. *Forensic science international*, *256*, 1-6.

Wang, M., Carver, J. J., Phelan, V. V., Sanchez, L. M., Garg, N., Peng, Y., . . . Bandeira, N. (2016). Sharing and Community Curation of Mass Spectrometry Data with Global Natural Products Social Molecular Networking. *Nature biotechnology*, *34*, 828–837.

https://www.nature.com/articles/nbt.3597#supplementary-information

Watrous, J., Roach, P., Alexandrov, T., Heath, B. S., Yang, J. Y., Kersten, R. D., . . . Raaijmakers, J. M. (2012). Mass spectral molecular networking of living microbial colonies. *Proceedings of the National Academy of Sciences*, *109*(26), E1743-E1752.

Woolner, V. H. (2017). *New Halogenated SecondaryMetabolites from Red Algae of the South Pacific*. (PhD), Victoria University of Wellington,

Zhou, H., Shaverdian, A. A., Jagadish, H. V., & Michailidis, G. (2009). *Multiple step social structure analysis with Cytoscape.* Paper presented at the 2009 IEEE Symposium on Visual Analytics Science and Technology.