Molecular diversity of the Soft Coral *Lobophytum* in Sabang Island

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Abstract. In our continuous concern of biodiversity relating to molecular structures in Sabang island, we found complex species of soft coral (genus *Lobophytum*). Molecular phylogenetic analysis on 82 specimens confirmed the presence of *Lobophytum crassum*, *Lobophytum verum*, and *Lobophytum rigidum*. On the other hand, the identification of chemical profiles was performed by spectroscopy analysis based on metabolomics approaches. As a result, we identified the chemical diversity of cembrane-type diterpenoids class from all specimens. Moreover, metabolomics analysis using feature-based molecular networking showed *L. crassum*, *L. verum*, and *L. rigidum* genes poses fundamental roles in the formation of chemical variations. Our results indicated that cembrane-type diterpenoids were found on all species related to genus *Lobophytum* in Sabang island, located in the western part of the Indonesian archipelago.

1. Introduction
Genus *Lobophytum*, known as soft coral; contains a rich sources of cembrane-type diterpenoids [1]. In nature, this class of molecules may act as a chemical defense against predators to ensure their protection and survival [2]. In addition, cembrane-type diterpenoids have a diverse pattern of structural variations with a multitude of functional groups and cyclizations to provide a variety of ring sizes, oxidation patterns, and the respective biological activities [3]. Previous research has shown that these class of molecules contain moderate cytotoxic properties against various cancer cell lines [4-8]. Regardless of Sabang island’s biodiversity concern, a considerable number of unique organisms were discovered [9-11] however, our attention concentrated on soft coral genus *Lobophytum*. Phylogenetic analysis (i) was applied to investigate the diversity of species, whereas the metabolomics approach (ii) provided chemical profiles of cembrane-type diterpenoids class and corresponding biomolecules.
2. Material and Methods

2.1. General experimental section
The secondary metabolites of all specimens were analyzed by Nuclear Magnetic Resonance (NMR) 500 MHz spectroscopy (Bruker Biospin, Billerica, MA, USA) in CDCl$_3$ (deuterated chloroform) solvent (Cambridge Isotope Labs). The LC-MS/MS data was subjected to reversed-phase analytical HPLC column (5µm, 250mm x 4.6mm). Furthermore, peaks were detected by positive ion mode of high resolution MS/MS system.

2.2. Biomaterial, extraction, and purification procedure
Samples were collected at displayed research sites during SCUBA expeditions, in shallow waters (<30m) surrounding Sabang island (Fig. 3). The collection of samples were conducted from March 2017 to April 2018. Freshly acquired specimens were extracted using acetone (3 x 2 L). After concentrating the extract, samples were partitioned by EtOAc : H$_2$O (v/v) collecting the organic layer for purification and metabolomics purposes. All crude extracts were purified using silica gel open column chromatography with n-hexane : EtOAc solvent systems. Additionally, target fractions were injected into normal-phase HPLC using n-hexane : acetone (100 : 12) solvent systems.

2.3. General procedure of phylogenetic analysis
To obtain DNA for phylogenetic analysis, all crude extracts (10.0 mg) were extracted using Qiagens’ DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), followed by PCR amplification using 16S-rDNA primer 16S-ar-L (5’CGCCTGTTATCAAAACAT-3’). Amplicons were analyzed by AB3130 with ABI PRISM BigDye Terminator Cycle Sequence Kit v1.1, and MEGA v5.1 for sequence alignment.

Figure 1. Sampling site near Sabang island
2.4. General procedure of chemical profile and Metabolomics approach

The presence of cembrane-type diterpenoids was confirmed by $^1$H NMR spectra comparison [12-18]. Meanwhile, a MeOH solution (20 μL) containing each crude extract was prepared for LC-MS/MS analysis. The flow rate was applied at 0.5 ml/min under positive ion mode of high resolution within a range of 50-1000 m/z. Raw data was converted to .mzXML format data, and is online available (reference number: MSV000086120, https://massive.ucsd.edu). MS/MS spectra were analysed by MZmine2 software. After analysis, MS/MS and quantitative tables were uploaded on the GNPS website (http://gnps.ucsd.edu) for feature-based molecular networking purposes. Parameters were acquired up to 10 minimum matched peaks with a cosine score of 0.6. The result was visualized by Cytoscape 3.7.2 version [22].

3. Results and Discussion

In 82 collected samples, the presence of Lobophytum crassum, Lobophytum verum, and Lobophytum rigidum was confirmed by molecular phylogenetics based on 16S-rDNA. Furthermore, crude extracts were subjected to open column and HPLC for purification purposes. As a result, known compounds of cembrane-type diterpenoids (Figure 1) were obtained from the genus Lobophytum [12-18]. Interestingly, there were also artificial products obtained (Figure 1, 8-11).

![Figure 2. Cembrane-type Diterpenoids Class from Genus Lobophytum](image)

Further analysis using LC-MS/MS confirmed isomeric molecules contain 5-membered lactone moiety (Table 1). The isomeric molecules (7) and 9 ($R_1$), and (10-14) suggest that the genus Lobophytum in Sabang island may embody a rich source of cembrane-type derivatives. In addition, the presence of artificial molecules containing hydroperoxide moiety (8) and double-bound migrations (9-11), confirming that singlet oxygen ($^{1}$O₂) was involved during purification steps.
On the other hand, molecular networking showed relations between biomolecules and matching chemical profiles. Since raw MS/MS data of 82 specimens was acquired, analysis by MZmine2 provided 1250 networking features. Moreover, these features were employed using the Global Natural Products Social web platform (http://gnps.ucsd.edu) to obtain a global figure of their metabolites [19-20]. This method is useful for rapid analysis, which investigates the metabolites by mass spectra data [21]. As a result, nodes introduced mass fragmentation MS/MS corresponding to individual molecular ions, which forms closely related clusters by feature-based molecular networking (Figure 2).

Table 1. LC-MS/MS result

| Compound # | Chemical Formula | Molecular Weight [M+H]+ |
|------------|------------------|-------------------------|
| 1          | C_{22}H_{30}O_{4} | 359.48545               |
| 2          | C_{26}H_{34}O_{10}| 507.55545               |
| 3          | C_{20}H_{31}O_{3} | 319.46472               |
| 4          | C_{22}H_{31}O_{6} | 319.48345               |
| 5          | C_{21}H_{33}O_{4} | 349.49045               |
| 6          | C_{22}H_{33}O_{4} | 361.50126               |
| 7          | C_{20}H_{32}O_{5} | 317.46882               |
| 8          | C_{20}H_{32}O_{5} | 349.44645               |
| 9 (R_{1})  | C_{20}H_{29}O_{3} | 317.45622               |
| 9 (R_{2})  | C_{21}H_{33}O_{4} | 345.45845               |
| 10         | C_{20}H_{29}O_{3} | 317.45904               |
| 11         | C_{20}H_{29}O_{3} | 317.44205               |
| 12         | C_{22}H_{31}O_{4} | 359.48895               |
| 13         | C_{22}H_{31}O_{4} | 359.48870               |
| 14         | C_{22}H_{31}O_{4} | 359.48882               |

Figure 3. Molecular Network of genus *Lobophytum*
Molecular networking of genus *Lobophytum* proposed that *L. crassum*, *L. verum*, and *L. rigidum* are responsible for producing cembrane-type diterpenoids 1-14. In addition, isomeric molecules (7, 9), and (10, 11, 13, 14) were determined as biomolecules closely related to each other, excluding compound 12 (Figure 2).

4. Conclusion

All specimens of genus *Lobophytum* produced cembrane-type diterpenoids class with 5-membered lactone moiety. The presence of artificial products succeeding purification, was confirmed by the presence of hydroperoxide (8) and double-bound migrations (9-11) derivatives. Further metabolomics analysis concluded that all complex species from *L. crassum*, *L. verum*, and *L. rigidum* are responsible for the production of secondary metabolites of cembrane-type diterpenoids class of the genus *Lobophytum* in Sabang island.

5. References

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