ANTHROPOGENIC EFFECTS ON WATER QUALITY, BIOACTIVE COMPOUND CONTENT, AND ANTICANCER ACTIVITY OF SOFT CORAL *Sinularia* sp. IN LIKUPANG WATERS, NORTH MINAHASA, INDONESIA

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ABSTRACT

The purpose of this research was to determine the anthropogenic effects of the waters of Likupang on water quality, the production of bioactive compounds from soft coral, and anticancer activity. Coral reef structure analysis is based on underwater photography with categories including sponges, soft corals, hard corals, sand fragments, and algae. CPCe analysis of nitrite, phosphate, ammonia, nitrate, pH, and dissolved oxygen was conducted via portable devices (HACH DR-890 and HACH HQ40d). Bioactive compounds from soft coral were extracted via 95% ethanol. To separate inorganic substances from bioactive compounds, reverse-phase flash chromatography was employed, with a C_{18} stationary phase and a mobile phase of methanol and dichloromethane. The bioactive compounds were separated via preparative high-performance liquid chromatography. Anticancer activity was evaluated via the MTT assay method with cervical cancer (HeLa) and breast cancer (MCF-7) cell lines. Water quality measurements revealed a phosphate concentration of 0.55 mg/L, a nitrate concentration of 0.13 mg/L, a nitrite concentration of 0.14 mg/L, an ammonia concentration of 0.18 mg/L, and individual composition features of 0–10% hard coral, 3.12–17.26% coral blossoms, 2.14–4.16% algae, and 0–20.42% fragments. Two compounds with anticancer activity were obtained from Sinularia sp., namely, flexilarin B, with an LC_{50} value of 0.98 mg/L for HeLa cancer cells, and episinulariade acetate, with values of 0.89 mg/L for MCF7 cells and 0.88 mg/L for HeLa cancer cells.

Keywords*:* Anthropogenic, Likupang, Soft coral, Anticancer, Bioactive compound

INTRODUCTION

Soft corals in coral reef areas are a source of germplasm that produces diverse potential secondary metabolite compounds in the field of biopharmology. Typically, these chemicals are synthesized by soft corals in response to ecological pressures to ensure their survival or secure their place in the benthic environment. Quantitatively, research on bioactive compounds in soft corals has revealed a connection between bioactivity and the production of bioactive compounds from soft corals with water quality[4,9]. The challenges posed by fellow benthic organisms in the coral reef

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habitat, or the diversity and complexity of its community, are crucial factors triggering the production of bioactive compounds in soft corals.

Anthropogenic pressures can lead to the degradation of coral reef cover complexity, such as the influx of waste into water, fishing activities, and shipping routes, posing threats to the sustainability of biodiversity and the alteration of coral cover patterns as substrates for soft coral [4,9]. Changes in water quality are highly vulnerable to coral reef diversity, but other organisms with relatively high resilience, such as macroalgae, soft corals, and sponges, can produce secondary metabolites with pharmacological potential[1].

Likupang waters are areas with diverse marine life that produce high levels of bioactive compounds, such as soft corals. However, high anthropogenic pressure, such as mining activities, can shift the pharmacological potential value of these biota. The eco-physiological response of bioactive cembranoid compound production from soft corals is crucial for understanding the impact of anthropogenic pressure on the bioactive content of soft coral biota in Likupang waters. Since the production patterns of bioactive compounds in soft corals are ecologically related to their environment (both biotic and abiotic), changes in the Likupang water environment are likely to affect the biopotential of bioactive compounds in soft corals. Differences in antropogenic pressure on the water quality of Bunaken Island and Manado Tua influence the content of bioactive compounds and the anticancer activity of soft corals [10]. This research evaluated water quality, benthic space competition, the characteristics of bioactive compounds in soft corals, and anticancer toxicity.

EXPERIMENTAL

Water and soft coral samples were collected at three sampling points: near the coastline, in the middle, and toward the open sea. Sample analysis and testing were conducted at the Biotechnology and Pharmaceutical Marine Laboratory, Faculty of Fisheries and Marine Sciences Sam Ratulangi University Manado, Indonesia.

This research is divided into several subactivities. First, the water quality patterns in Likupang waters were examined, followed by the characterization of bioactive compounds in soft corals, and finally, the anticancer activity was tested. The cancer cell lines used were cervical cancer (HeLa) and breast cancer (MCF-7) cells. For water quality analysis, water samples of the selected parameters were collected at the same depth. Water variables, including nitrite, ammonia, phosphate, nitrate, pH, and dissolved oxygen, were chosen to assess the level of anthropogenic impact. The chosen parameters are crucial indicators of the ecological health of the marine environment and can be used to assess the potential influence of anthropogenic activities on water quality. All the variables were analyzed onsite via portable devices (HACH DR-890 and HACH HQ40d) to ensure immediate and accurate results.

The methodology for water quality analysis is outlined as follows:

The water samples were collected at a consistent depth. The samples were collected at three designated points: near the coastline, in the middle, and toward the open sea. The collected water samples were analyzed directly in the field with portable devices. Real-time (on-site) analysis was

carried out with these devices to measure the reliability and efficiency of the selected water parameters.

Sample collection and preservation. Soft coral biota were collected from Likupang waters at a depth of 5 m via SCUBA diving techniques for safe and precise collection. Taxonomic identification of soft corals was performed by examining their morphology via the prescribed methods[2].

The preservation procedure consisted of immersion 500 g of the collected soft coral samples in 1 L of ethanol. The ethanol-immersed samples were then placed in an ice-cooled box for preservation during transportation from the field to the laboratory. A cool box maintained at a low temperature with the inclusion of ice was used to ensure the preservation of the soft coral samples during transportation.

This meticulous sampling and preservation process aims to maintain the integrity of the soft coral samples and their taxonomic information, enabling accurate subsequent analyses in the laboratory. The use of ethanol and cold storage helps prevent degradation of the samples and ensures the reliability of the data collected during the fieldwork.

Extraction and Isolation of Bioactive Anticancer Compounds

In the laboratory, the sample was subjected to triple extraction. The entire extract (3 L) was subsequently filtered and dried via an evaporator (Buchi 250 Rotavapor) and concentrator. The resulting dry extract was then filtered from inorganic substances via reverse-phase flash chromatography, with a C_{18} stationary phase (Phenomenex C_{18}) and a mobile phase of dichloromethane and methanol (1:1). The solvent from the filtration mixture was dried again via an evaporator and concentrator and then reconstituted with 4 mL of methanol.

The isolation of bioactive compounds was carried out via preparative high-performance liquid chromatography (HPLC) (Shimadzu HPLC Preparative with a Shimadzu C_{18} column measuring 250 × 21 mm). The mobile phase used was a gradient elution from 20% acetonitrile/water to 100% acetonitrile over 60 minutes at a flow rate of 15 mL/min. Fractionation of the sample was performed automatically via a fraction collector every 30 minutes.

The results from each fraction were then qualitatively analyzed against cancer cells via the 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolinone bromide method[12]. Powdered extract samples were weighed and then dissolved in DMSO to a concentration of 100,000 ppm. The stock test solution was subsequently diluted to 10,000 ppm and further diluted to 1000 ppm. The testing dose series used was 30 ppm, 15 ppm, 7.5 ppm, 3.75 ppm, 1.875 ppm, 0.937 ppm, and 0.468 ppm. The determination of these doses was performed with the chemotherapy drug doxorubicin at 30 ppm. The test solution was added to the culture media to a volume of 1000 µL. Next, the cells were centrifuged for 5 min at 2000 rpm, the supernatant was discarded, the pellet was resuspended in 4 mL of culture media, the cells were slowly suspended, and 2 mL of suspended cells was subsequently transferred to a 25 cm^3 culture flask. The cells were grown in an incubator with a CO_2 flow of 5 ml/minute at 37°C for 12 hours.

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Once the cells reached approximately 80% growth, the culture flask was emptied of media, and the cells were rinsed twice with 2 ml of phosphate-buffered saline. Next, 500 µl of a 0.25% trypsin-EDTA enzyme mixture was added, and the cells were then placed in a CO_2 incubator for 4 minutes. After this incubation period, the cells were carefully removed from the flask and added to fresh culture media, where they were gently mixed until they detached from the flask's base media. The cells are moved to a conical tube. A total of 10 µL of cells was transferred into an Eppendorf tube and mixed with 90 µl of complete medium via a micropipette to create a suspension. Next, a volume of 10 µl of cells was extracted and introduced into a hemocytometer. The number of cells was subsequently ascertained by viewing them under a microscope and with a counter. The cells were enumerated in four hemocytometer chambers, with dark-colored cells considered nonviable and hence excluded from the count. The calculation for determining the quantity of cells per millilitre was performed via the following formula:

Total cells=

$$\frac{(\sum \text{cells counted in chamber A})+(\sum \text{cells counted in chamber B})+(\sum \text{cells counted in chamber C})+(\sum \text{cells counted in chamber D})}{4} \times 10^4$$

The number of cells required for this study was 20×10^4 cells/well × 100 wells

Chemical structure analysis of active anticancer compounds

Elucidation of the structure of bioactive isolates

The process of identifying bioactive compounds involves analyzing the chemical shift data of each proton and carbon molecule via NMR instrumentation (specifically, a Jeol ECS 400 MHz). Additionally, molecular weight data and UV–Vis absorption data were examined via LC–MS instrumentation (specifically, Shimadzu Hybrid Ion Trap - Time of Flight).

Molecular weight analysis via LC-MS and FT-MS methods

The active chemicals are solubilized in 100 µL of methanol and subsequently introduced into the LCMS instrument by injection. The LCMS system consists of a reversed-phase C18 silica stationary phase (Prep ODS Shimadzu 250 mm × 20 mm) and operates with a mobile phase flow rate of 15 mL/minute. The mobile phase composition followed a gradient method, starting with 10% acetonitrile in water and gradually increasing to 100% acetonitrile over a period of 20 minutes. This was followed by isocratic elution with 100% acetonitrile for 10 minutes. The total duration of the mobile phase method was 30 minutes. The LCMS system is equipped with a DAD (Diode Array Detector) detector, which operates in the wavelength range of 200–800 nm. Additionally, an accurate mass detector is also used.

Measurement of the Proton and Carbon Chemical Shift Data of the Active Compounds

The isolate (10 mg) was dissolved in deuterium chloroform and deuterium methanol. The sample was placed in NMR tubes and then analyzed in one dimension ('H and '3C) or two dimensions (HMBC, COSY, HMQC), with tetramethylsilane (TMS) used as a chemical shift standard.

Determination of Data from Spectroscopic Analysis

The determination procedure commences by searching for active compounds via the combined molecular weight data obtained from LC–MS and FT–MS, along with UV–VIS absorbance and NMR data, in the MarineLit 2014 electronic database. If no compounds that match are located in the database, the active chemicals are deduced on the basis of the general pattern of spectroscopic data gathered.

Correlation between water quality and bioactive compound activity

Descriptive analysis of bioactive compound yield:

The average quantity of bioactive compounds obtained in the previous subsection was analyzed descriptively. Multivariate analysis was employed to investigate the correlation between water quality and sample bioactivity. The initial steps involved categorizing the IC_{50} values, where samples with inhibition values greater than 60% at 100 ppm were categorized as "high," those with inhibition values between 20% and 60% were categorized as "moderate," and those with inhibition values less than 20% were categorized as "low." The correlation between bioactivity categories and local coverage was conducted via canonical discriminant analysis on the basis of method[7].
Statistical analysis was performed via Past Statistical Software v3.08. Anticancer toxicity analysis was performed via probability analysis with the Minitab version 15 statistical program.

LC_{50} determination:

Cytotoxicity testing involves the use of MCF-7 breast cancer cells and HeLa cervical cancer cells. The cells were cultivated in fully supplemented RPMI 1640 medium, specifically Roswell Park Memorial Institute 1640, which consisted of 10% fetal bovine serum. The cultivation was carried out in a CO2 incubator at 37°C. The MTT test, which involves the use of 3-(4,5-dimethylthiazol-20 yl)2-5-diphenyltetrazolium bromide, was employed. The total cytotoxicity test involved culturing 10,000 cells/well, following the modified guidelines[12]. The absorbance of each well was measured with a microplate reader at wavelengths of 570 and 690 nm. The percentage of cell death was calculated via the following formula:

Cell death (%) = ─[(xD-xA) - (xB-xC)/(xD-xC)] × 100%.

xA = absorbance of the control cell
xB = absorbance of the cell extract
xC = absorbance of the control extract
xD = absorbance media control

Data analysis

This integrated approach combines descriptive analysis, multivariate correlation studies, and specific toxicity evaluations to understand the relationship between water quality and the bioactive properties of the isolated compounds.

RESULTS AND DISCUSSION

Water quality in the Likupang coral reef area

The Likupang waters in North Minahasa have experienced a decline in water quality. The high levels of phosphate, nitrate, nitrite, and ammonia, along with the low pH of the observed water indicated a decrease in water quality. The degradation is attributed to the increased discharge of wastewater, as well as the weathering of rocks. Residential waste contains domestic waste, including organic and inorganic waste, as well as detergent. Organic waste consists of biodegradable materials such as leftover vegetables, fruits, and leaves, whereas inorganic waste includes items such as paper, plastic, glass, fabric, wood, metal, rubber, and leather, which are nonbiodegradable. The low pH is a result of elevated organic pollution caused by aerobic decay by bacteria, leading to the production of carbon dioxide, pH reduction, and the inflow of mining.

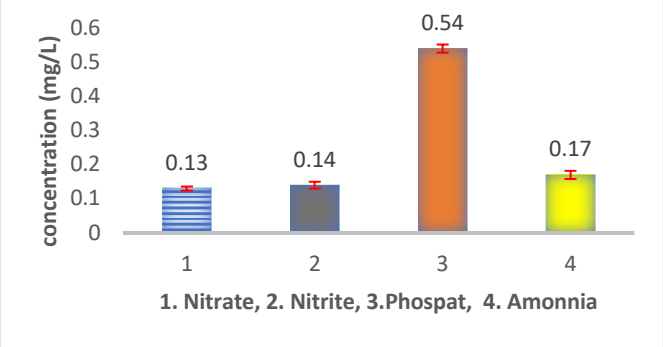


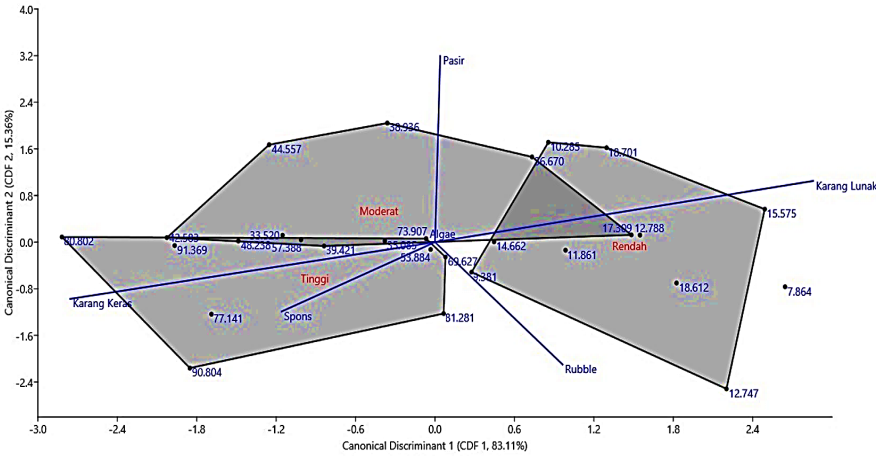
Figure 1. Characterisitic of water quality to Likupang waters

Ammonia is a parameter indicating organic pollution in water generated through the anaerobic decomposition of organic materials (eutrophication) by microbes[4]. A high ammonia content in water can result in turbidity and unpleasant odors[15,19]. Phosphate (PO_4) is naturally present in minimal amounts in water and plays a role as both a mineral and organic compound. An increase in phosphate levels poses a danger to aquatic organisms [15]. In natural aquatic environments, the phosphorus content is typically 10%, with the remaining 90% originating from human activities such as industrial and domestic waste disposal. Elevated phosphate levels in water can lead to eutrophication[19,20]. While phosphate is an essential nutrient for aquatic organism growth, its high concentration indicates contamination. Phosphate compounds generally originate from industrial waste, fertilizers, domestic waste, and the breakdown of other organic materials. The presence of nitrate, nitrite, phosphate, and ammonia is illustrated in Figure 1. Republic of Indonesia government regulation number 21 of 2021 concerning water quality for the growth of marine organisms where nitrate is 0.06 mg/L, phosphate 0.015 mg/L and ammonia 0.3 mg/L. Compared

with the analysis data, there was a decline in water quality in Likupang waters with an increase in several measured parameters.

Characteristics of water quality in Likupang Waters

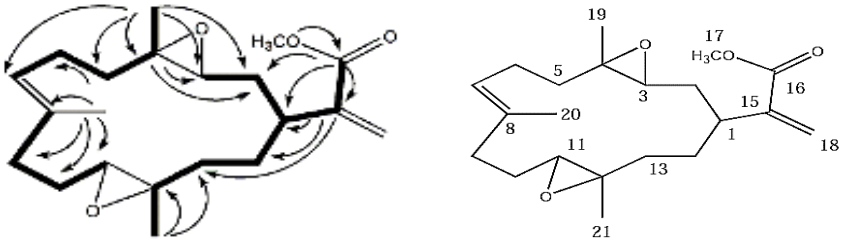
The decrease in water quality has led to a reduction in hard coral cover, resulting in the invasion



of noncoral biota. *Sinularia sp*., which resembles soft coral, is highly prevalent in coral reef ecosystems. Analysis of the anticancer potency plotted against benthic space competition from samples indicates that benthic space competition is a major factor causing a decrease in the cytotoxicity of the samples. In areas with low benthic competition, characterized by high coverage of soft corals, rubble, and sand as abiotic components, a decrease in the production of bioactive compounds from soft coral organisms may occur. Conversely, in areas with benthic competition involving hard corals and sponges, there is a potential increase in anticancer activity. The presence of anthropogenic pollution is part of the main threat to the production of natural ingredients from soft coral, which have biopotentials in the field of pharmacology[5]. The relationship between benthic space competition and the anticancer activity of soft corals is illustrated in Figure 2 below

Figure 2. Benthic Space Competition and Anticancer Activity of Soft Coral

Analysis of the bioactive compounds from the soft coral *Sinularia* sp. yielded two known compounds. The first is *Flexilarin*-B, with the structural formula $C_{12}H_{32}O_4$ and a molecular weight of 348.23. The second is *episinularide acetate,* with the structural formula $C_{22}H_{23}O_4$ and a molecular weight of 376.2, as per the Marilite 2004 database. Typically, the chemical structures produced by soft corals are in the form of cembranoid terpenoids. Structural characterization was performed on the basis of the mass spectrometer pattern and 1D/2D-NMR, with spectra referenced to the resonance of 1H and 13C in a deuterated solvent. The chemical structures obtained are shown in Figure 3 below.



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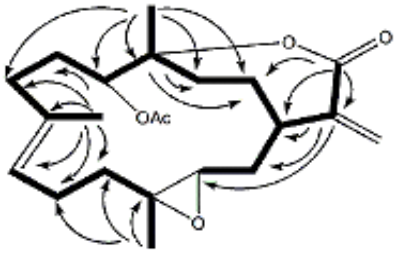
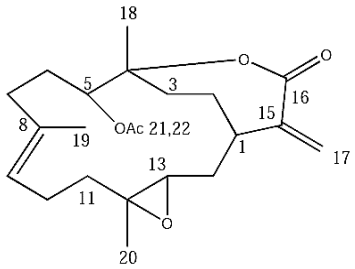
a). Flexilarin B Compound Group Structure Formula $C_{21}H_{32}O_4$ BM 348,23





b). Episinularide acetate compound group structure formula $C_{22}H_{23}O_4$ BM 376.2

Figure 3. Chemical structures of the anticancer compounds from *Sinularia* sp.

The flexilarin-B compound has a methoxide group and a ketone group at the C16 atom, and there is a double bond between C6 and C7. On the other hand, the episinularide acetate compound has an acetate group at C21, a ketone group at C16, and a double bond between C7 and C8. The chemical structure model that was discovered is typically synthesized by soft corals[3,16,17]. The NMR spectrum data for the two pure anticancer activity compounds are provided in Table 1 below.

Table 1: NMR spectra and active anticancer compounds from *Sinularia sp*. soft coral

No	Compound a		Compound b	
	δ_C	δ_H (Mult,J in Hz)	δ_C	δ_H (Mult,J in Hz)
1	33.2	2.43 (m)	36.184	2.23 (m)
2	29.7	2.19,2.17(m)	29.891	1.98,1.42(m)
3	62	2.9(t.13.7)	31.71	2.12,1.72(m)
4	60.4		80.72	
5	36.2	2.16,1.23(m)	92.962	3.511(m)
6	23.1	2.35,2.22(m)	22.769	2.76,2.62(m)
7	124.7	5.14(d.10.8)	34.525	1.49,1.54(m)
8	132.9		144.2	
9	33.9	2.22,2.15(m)	125	5.12(t,10.1)
10	29.3	2.05,1.38(m)	22.3	1.64,2.17(m)
11	66.6	4.1(d.9.6)	37.28	2.53,2.37(m)
12	58.7		61.765	
13	29.4	2.31,1.83(m)	64.749	2.972(t,14.2)
14	31.1	1.98,1.46(m)	31.731	1.91,1.29(m)
15	142.4		145.936	
16	167.3		169.859	
17	48.8	3.5(s)	125.685	6.321,5.434(d)
18	122.3	6.3,5.3(s)	18.087	1.61(s)
19	14.1	1.21(s)	15.799	1.25(s)
20	13.9	1.61(s)	15.904	1.42(s)

21	20.9	1.33(s)	172.2	
22	-		56.33	3.52(s)

The Flexilarin-B compound exhibited anticancer activity, with an LC_{50} of 0.98 mg/L for MCF-7 cells, resulting in a cell mortality rate of 83.05%, and 0.99 mg/L for HeLa cells, with a cell mortality rate of 80.91%. The compound episinularide acetate has an LC_{50} of 0.89 mg/L for MCF7 cells, with a cell mortality rate of 81.05%. According to previous study[11], an extract can be considered to have anticancer properties if the cytotoxic value for the LC_{50} is less than 1000 ppm, and for compounds, the LC_{50} is less than or equal to 30 ppm. These findings suggest that the smaller the IC_{50} value is, the more toxic the compound is. On the basis of the criteria of the National Cancer Institute (NCI), if the IC_{50} of an extract is less than 20 µg/mL, it is considered active as an anticancer agent[12]. Secondary metabolites from soft coral have anticancer activity[3,17,3]. A new compound from the soft coral *Sinularia* sp. from Bunaken Island named *unsrat sinularin,* which exhibited anticancer toxicity against MCF-7 and HeLa cells[10] was identified. A bioactive compound extract from the soft coral Sinularia sp. has strong cytotoxic effects on MCF-7 cells[14]. The chemical structure of the soft coral *Sinularia querciformis* from Taiwan, which has anti-inflammatory and anticancer activities, was determined[19].

CONCLUSION

The environmental conditions in the Likupang waters of North Minahasa have been under anthropogenic pressurewhich has resulted in a decrease in biodiversity. Two compounds were obtained from the soft coral *Sinularia sp*., namely, Flexilarin B, which has anticancer activity, and an LC_{50} of 0.98 mg/L for MCF-7 cells, with cell mortality rates of 83.05%, and 0.99 mg/L for HeLa cells, with a cell mortality rate of 80.91%. The compound episinularide acetate has an LC_{50} of 0.89 mg/l for MCF7 cells, with a cell mortality rate of 81.05%, and an LC_{50} of 0.88 mg/l for HeLa cells, with a cell mortality rate of 81.01%.

CONFLICT OF INTEREST

The author states that there is no conflict between this article

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