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Original Research Article

The Antibacterial and Anti-Breast Cancer Activities of *Caulerpa racemosa* from the Minahasa Peninsula Water and North Sulawesi, Indonesia

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ABSTRACT

Caulerpa racemosa is a type of green algae recognised for its ability to inhibit the growth of bacteria and its potential to combat breast cancer. The algae are composed of several bioactive chemicals, such as flavonoids, phenols, tannins, saponins, steroids, alkaloids, and terpenoids. Hence, this study aimed to ascertain the antibacterial and anti-breast cancer properties of *Caulerpa racemosa* extract obtained from the Minahasa Peninsula, notably the North and Southeast water regions. The anticancer test assessed the algae extract cytotoxicity against MCF-7 breast cancer cells using the MTT assay for direct measurements. The antibacterial test employed the Kirby Bauer disc diffusion method. The findings indicated that the extract of *Caulerpa racemosa* collected from the water in North Minahasa exhibited a potent antibacterial activity. The substantial antibacterial action of the substance was demonstrated by an average inhibition zone of 13.5 mm against *Mycobacterium smegmatis* at a concentration of 1 mg/mL, the highest concentration tested. The extract from water in the North and Southeast Minahasa regions exhibited significant anticancer activity, with IC_{50} values of 149.9 ppm and 313.2 ppm, respectively.

Keywords: *Caulerpa racemosa*, Antibacterial, Anti-Breast Cancer, Minahasa Peninsula Water

Introduction

The waters of North Sulawesi, especially around the Minahasa Peninsula, possess abundant marine resources, making it an attractive tourist spot and an important research area. The presence of macroalgae in this water is essential for preserving the equilibrium and well-beingof the ecosystem.[1] These algae exhibit a high growth capacity, contain chlorophyll and carotenoids, and produce colonies of many cells abundant in organic substances such as polysaccharides, hormones, vitamins, minerals, and bioactive compounds.[2] *Caulerparacemosa* possesses a variety of secondary metabolites, such as flavonoids, phenols, tannins, saponins, steroids, alkaloids, and terpenoids, with therapeutic uses. Moreover, phenols can function as antioxidants within the body, thereby inhibiting the occurrence of degenerative ailments such as premature ageing, cancer, cataracts, heart disorders, and diabetes.[3,4] The prevalence of health disorders caused by infections is significantly high in Indonesia. Viruses, bacteria, and fungi are responsible for causing diseases in humans, which can have lethal implications if not effectively treated.

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Furthermore, *Salmonella typhi*, a gram-negative pathogenic bacterium, is known to cause typhoid fever and can be acquired by consuming food contaminated due to unsanitary conditions.[5,6] Tuberculosis, an extremely contagious illness, is caused by *Mycobacterium tuberculosis*, *M. bovis*, *M. kansasii*, and *M. africanum*, which readilytransmit through the air. Nevertheless, *Mycobacterium smegmatis* is suggested as the preferredbacteria because it resembles *M. tuberculosis*.[7,8] Another common health concern is breast cancer, which is characterised by the abnormal and uncontrolled proliferation of cells in the breast region. In 2018, there were a total of 2,088,849 instances of breast cancer worldwide, which accounted for 11.6% of all reported cases. Out of these cases, there were 626,679 reported deaths, making up 6.6% of the total number of deaths.[9] The utilisation of marine natural resources, specifically the macroalgae *Caulerpa racemosa*, for therapeutic applications is anticipated to produce a superior outcome compared to synthesised medications, owing to its potent secondary metabolites. This study aimed to ascertain the antibacterial and anti-breast cancer properties of *Caulerpa racemosa* extract obtained from the Minahasa Peninsula (North and Southeast water regions) of Indonesia.

Materials and Methods

Reagents, glass wares, and equipment

These include IKA RV 10 rotary evaporator, diving equipment, autoclave, incubator, ultraviolet lamp, chamber, micropipette, balance, gloves, scissors, knife, Erlenmeyer flask, measuring glass, beaker/chemical glass, 15 cm Petri dishes, spatula, forceps, microtubes, stir bar, refrigerated cabinet, urine container, calliper, vials, hypodermic needle, lab coat, glass vessel, reaction tubes, 8 mm Advantec paper discs, camera, jars, and aluminium foil. Bio Safety Cabinet (BSC), centrifuge, CO_2 incubator, microscope, multimode reader, hemocytometer, 1.5mL microtubes, 15 mL tubes, 75 mL T-flasks, and a 96-well plate. Others included 95% ethanol, 70%

alcohol, n-hexane, ethyl acetate, 8-diameter filter paper discs, distilled water (asa control), ethyl acetate, octadecyl silica, Middlebrook ADC growth supplement, McFarland standard set R092-1NO and streptomycin sulfate. Cisplatin, antibiotics, dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), PrestoBlueTM Cell Viability Reagent, Roswell Park Memorial Institute Medium (RPMI), fetal bovine serum (FBS), trypsin-EDTA, trypan blue, and 75% ethanol were also used.

Microorganism: *Mycobacterium smegmatis*

Plant collection and preparation

Caulerpa racemosa (Forssk) J.Agardh sample was collected from the Minahasa Peninsula (North: 1°24′9″N 124°57′36″E and Southeast Minahasa Regencies: 1.0279° N, 124.7299° E) in the North Sulawesi Province (Figure 1). The sample was identified in the plant taxonomy section of the basic biology laboratory, Faculty of Mathematics and Natural Sciences, Sam Ratulangi University, Manado, with voucher number 56/LBD.07/IT/2024. The algae specimens (Figure 2) were obtained from the natural environment and rinsed with a continuous flow of purified water to remove any attached residues or debris. The samples were allowed to dry, cut into smaller pieces, pulverised, and weighed using an analytical balance.

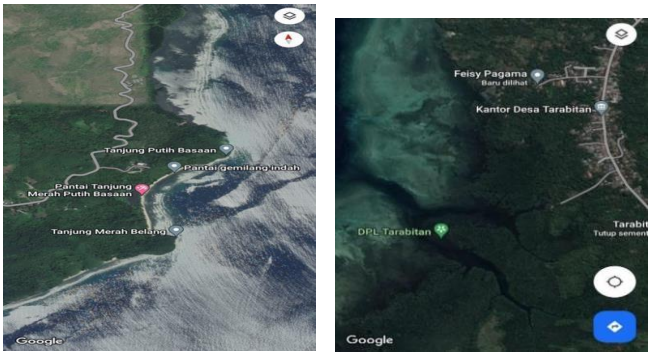

Figure 1. Sampling Area in Southeast and North Minahasa Water

Plant Extraction

About 402 and 450 g of *Caulerpa racemosa* from North and Southeast Minahasa, respectively,were separately soaked in 2 L of 95% ethanol for 24 hrs. The marc was re-extracted 3 times with fresh solvents of the same volume. The resulting extracts were filtered using filter paper.The combined filtrates were evaporated using a rotatory vacuum evaporator to get the concentrated extract.

Column Chromatography

The chromatographic column was filled with cotton soaked in methanol, and then 40 g of octadecyl silica slurry was added and left to soak in methanol overnight. The concentrated extract was loaded onto the octadecyl silica column and eluted with a gradient mixture of methanol and water (20%, 40%, 80%, and 100%). The fractions were separated based on their concentrations and collected in Erlenmeyer flasks, then evaporated to dryness using a rotary evaporator. The concentrated extracts were kept in labelled vials.

Anticancer Test

The anticancer screening test followed the stages below:

a. Media/Positive Control/Sample Preparation: A liquid culture media, Roswell Park Memorial Institute Medium (RPMI), was prepared. The media was supplemented with 10%Fetal Bovine Serum (FBS) and 50 µL/50 mL of antibiotics. Cisplatin was employed as the positive control. In addition, the samples were dissolved to form a concentrated solution, and PrestoBlue™ Cell Viability Reagent was used to make the working solution for the anti-proliferation assay.
b. Cell Preparation (MCF-7): Cells with a confluence of at least 70% were subjected to the following steps: the media was removed from the dish and washed two times with 1 mL ofPBS, then 1 mL of Trypsin-EDTA solution was added and incubated for 5 minutes (cells were observed to float under an upside down microscope). The scattered cells were movedto a liquid-filled tube and spun at a speed of 3000 revolutions per minute for 5 minutes. Thesupernatant was discarded, and the solid portion was dissolved in a tube holding a liquid medium.
c. Placing cells into a 96-well plate: The quantity and viability of cells were assessed by trypanblue exclusion. MCF-7 cells with a final density of 170,000 cells/mL were suspended in media at a concentration of 17,000 cells per well. Furthermore, a sterile microtube was madewith 10 µL of trypan blue, to which 10 µL of the cell suspension was added and then mixedthoroughly. The hemocytometer was sterilised, and the slide was coated with 70% ethanol.A volume of 10 µL of cell-trypan blue solution was gradually introduced to a specific chamber area with a pipette. Subsequently, the number of viable cells in millilitres was assessed and calculated.
d. Cells Treatment. A total of 8 microtubes with a volume of 1.5 mL each were manufactured and labelled according to the necessary dilution concentration. The stock samples were diluted into 8 different concentrations using the media solvent. Subsequently, the 96-well plate holding the cells was removed from the incubator. The left edge was annotated to identify the rows subjected to standards and samples, and the medium was removed from each well. 100 µL of each sample and the positive control Cisplatin were placed into the appropriate wells of the 96-well plate containing cells using a micropipette. The cells werethen incubated for an additional 24 hours.
e. The PrestoBlue Reagent was added, and the absorbance was measured. The medium in eachwell was removed, and 9 mL solution was made in a tube, to which 1 mL of "PrestoBlueTM Cell Viability Reagent" was added (10 µL of reagent for every 90 µL of media). In addition,a 100 µL solution of PrestoBlue Cell Viability Reagent was introduced into each well of the microplate and left to incubate for 1-2 hours until a noticeable alteration in colour was detected. The addition of the reagent caused the blue chemical resorufin in PrestoBlue® to be converted into a highlyluminous red form. The converted value was directly proportional to the number ofmetabolically active cells and was determined quantitatively. The absorbance was determined by utilising the spectra of resazurin and resorufin. Afterwards, the amount of light absorbed was determined at a specific wavelength of 570 nm (with a reference wavelength of 600 nm) using a multimode reader.

Figure 2: *Caulerpa racemosa*

Equipment Sterilisation
All equipment for microbial screening was sterilised with an autoclave for 30 minutes at 121°C under 1 atmospheric pressure before media preparation.[10]

Antimicrobial Screening of plant extracts
The antimicrobial testing was conducted at the Pharmaceutical Biology Laboratory in the Faculty of Mathematics and Natural Sciences at the Indonesian Christian University of Tomohon from December 2022 to June 2023.

Preparation of Bacterial Suspension (Mycobacterium smegmatis)
Mycobacterium smegmatis was cultivated in 0.52 g of Middlebrook 7H9 liquid growth media, supplemented with 0.44 millilitres of glycerol and 99.56 millilitres of ADC, in a total volume of 100 millilitres. The bacteria was incubated for 48 hours in an incubator at a temperature of 37°C.[12] Streptomycin sulphate at a concentration of 2 µg/disc was used as the positive control agent.

Media Preparation
Middlebrook 7H10 (1.95 g) was measured and combined with 0.5 millilitres of glycerol. The mixture was then diluted with distilled water to a final volume of 90 millilitres. Afterwards, the solution was made uniform and free from microorganisms by homogenisation and sterilisation in an autoclave for 30 minutes at a high temperature of 121°C.

Antibacterial Test Procedure
The antibacterial test utilised a chromatography column-purified extract of *Caulerpa racemosa* with a purity level of 80%. Precisely 0.060 g of extract was weighed and then dissolved in 600µL of a solution containing 95% ethanol. Meanwhile, the positive control (streptomycin sulphate) was 2 µg/disc, and the negative control was 11 µL. The concentrations of the fractions of *Caulerpa racemosa* ranged from 200 to 1000 µg/disc. In addition, the filter paper discs underwent a two-day vacuuming process to guarantee that any ethanol absorbed on the discs was thoroughly dried, preventing any potential impact on the antibacterial capabilities. The bacterial suspension was combined with 1 mL of sterile media, and then 100 mL of medium was added to each Petri dish, which was then allowed to harden. Paper discs saturated with extract were likewise placed on the designated test media in labelled Petri plates and incubated for 48 hours at a temperature of 37°C.
The inhibition zones were calculated using the following formula.[11]
$D = \frac{A+B+C}{3}$
Description: A = vertical diameter
B = horizontal diameter C = diagonal diameter
D = inhibition zone diameter

Statistical analysis
The data obtained from the experiments were analysed using Microsoft Excel, an ELISA reader, and GraphPad Prism version Version 9, 2020.

Results and Discussion

The cytotoxicity of *Caulerpa racemosa* extract, derived from water samples collected from North and Southeast Minahasa, was evaluated against MCF-7 breast cancer cells. The positive control agent was cisplatin, while DMSO was the negative control. Cisplatin is a chemotherapeutic medication in the platinum-based class of anticancer agents. The cytotoxicity test against breast cancer cells is an *in vitro* assay that utilises cell cultures to assess the harmful effects of a chemical. The *in vitro* test is characterised by its rapidity, limited usage of chemicals, absence of test animals, and ability to provide insights into the possible impacts on human target cells.[13] Before quantifying absorbance, the crystals were dissolved using an organic solvent (water-soluble tetrazolium salt (WSTS)) to assess cell proliferation and cytotoxicity. The staining findings depicted in Figure 3 exhibit two distinct colours on the 96-well plate: a deep shade and a gradually diminishing orange hue, utilising eight different doses. The formazan crystals in each plate and their corresponding concentration result from a reaction between MTT salt and the oxidoreductase enzyme found in live cells. Dissolving these crystals in tetrazolium salt leads to the rupture and impairment of cells. Increased cellular damage leads to a transition from a dark to a gradually diminishing orange colour, as depicted in Figure 3(a) for the specimen obtained from Southeast Minahasa. Nevertheless, the fading shown in Figure 3(b) for the sample from North Minahasa was less pronounced. Both the positive and cell controls exhibit a dark orange colour, indicating the absence of any cell injury, as depicted in Figure 3(c). The 96-well plate undergoes colour changes, which were detected by measuring its absorbance using an ELISA reader. Observations were carried out using an ELISA reader at a wavelength of 600 nm, specifically for the orange colour at a length of 450 nm. ELISA, an enzyme-linked immunosorbent assay, is a widely used serological test in immunology laboratories. It involves the use of enzyme-labeled secondary antibodies. The light spectrum consists of primary and complementary colours that an object absorbs and may be seen without optical aids. Colour alterations are employed to perceive cell growth visually. Meanwhile, cells that undergo proliferation in mitochondria take in MTT, creating an orange colour caused by formazan crystals. The colour intensity is directly related to the number of viable cells.[14]

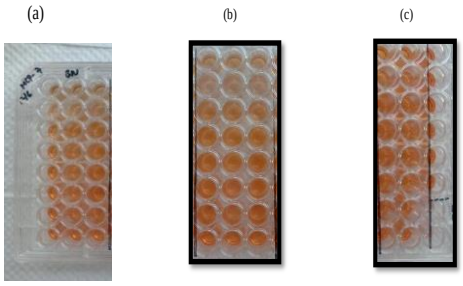

Figure 3: Staining Results of Southeast Minahasa *Caulerpa racemosa* (a), North Minahasa *Caulerpa racemosa* (b), Positive Control, and Cell Control (c)

Figure 4 demonstrates cell growth under a microscope by analysing the colour changes in the extract of *Caulerpa racemosa* obtained from North and Southeast Minahasa. The data indicates that the survival rate at a concentration of 7.81 ppm was 99.77%, 89.34%, and 95.22%; at 15.63 ppm it was 94.52%, 94.61%, and 94.48%; at 31.25 ppm it was 90.44%, 94.74%, and 96.45%; at 62.5 ppm it was 91.98%, 77.92%, and 82.11%; at 125 ppm it was 68.82%, 66.78%, and 66.65%; at 250 ppm it was 63.24%, 60.19%, and 63.26%; at 500 ppm it was 29.82%, 32.48%, and 28.23%; and at 1000 ppm it was 1.69%, 6.32%, and 6.54%. To enhance clarity, the data was transformed into a graphical representation in the form of a curve, as depicted in Figure 3. Concentrations ranging from 7.81 ppm to 1000 ppm exhibit different levels of cytotoxicity, with the least and most cytotoxic concentrations being 7.61 ppm and 1000 ppm, respectively. The linear regression value for this relationship was 0.9369.
The survival rates of organisms exposed to different concentrations of *Caulerpa racemosa* extract from the water of North Minahasa are as follows: at a concentration of 7.81 ppm, the survival rates were 99.01%, 94.017%, and 94.05%; at 15.63 ppm, the survival rates were 93.71%, 95.06%, and 97.12%; at 31.25 ppm, the survival rates were 92.36%, 103.76%, and 92.00%; at 62.5 ppm, the survival rates were 75.15%, 56.76%, and 83.94%; at 125 ppm, the survival rates were 60.87%, 59.68%, and 57.37%; at 250 ppm, the survival rates were 33.11%, 31.62%, and 29.85%; at 500 ppm, the survival rates were 1.83%, 2.03%, and 4.40%; and at 1000 ppm, the survival rates were 5.30%, 8.96%, and 4.77%. The above values are presented in Figure 5. The cytotoxic potency of the extracts fluctuates at concentrations ranging from 7.81 ppm to 1000 ppm.

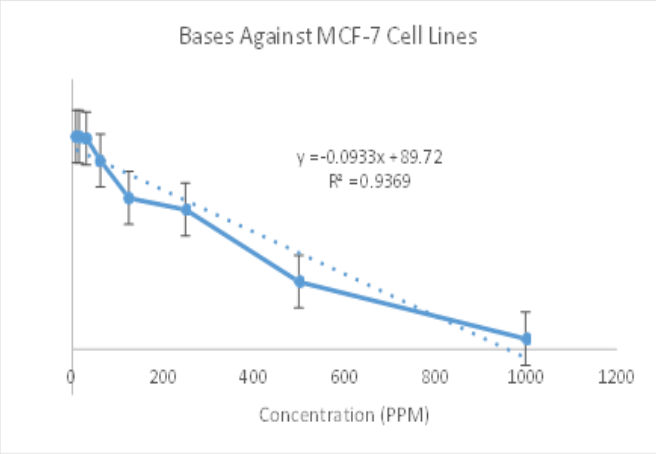

Figure 4: Cytotoxicity Test Curve of *Caulerpa racemosa* Extract from Southeast Minahasa

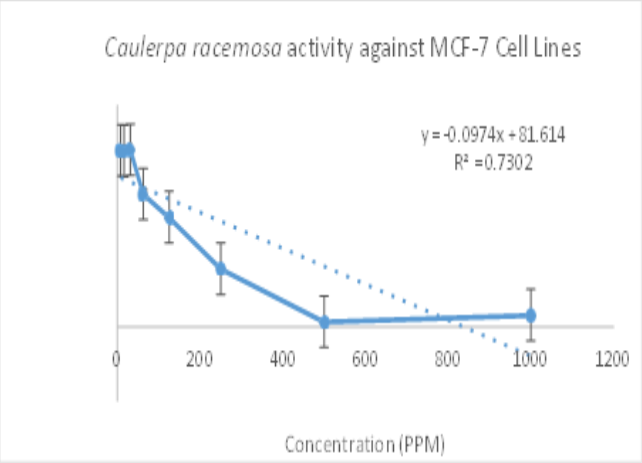

Figure 5: Cytotoxicity Test Curve of *Caulerpa racemosa* Extract from North Minahasa

The lowest toxicity was observed at 7.61 ppm, while the highest cytotoxicity was observed at 500 ppm. The linear regression value for this relationship was 0.7302. The IC_{50} value is the concentration of a material that inhibits cell proliferation by 50%. It is frequently employed as a metric in cytotoxicity experiments.[15] The IC_{50} values for the sampletaken from Southeast and North Minahasa water were 313.2 ppm and 149.9 ppm, respectively, as determined using nonlinear regression analysis of four factors. The IC_{50} value was established by plotting a graph that shows the percentage of cell survival against the concentration of the sample. The cytotoxicity of an extract against cancer cells can becategorised as highly potent, potent, moderately potent, or weak based on the IC_{50} value. A value below 10 µg/mL indicates high potency, while a range of 10-100 µg/mL indicates potency. A 100-500 µg/mL range suggests moderate potency and a value beyond 500 µg/mLindicates weak potency. The conversion factor for 1 ppm is 1 µg/g or 1 µg/mL. Therefore, 313.2 ppm is equivalent to 313.2 µg/mL, and 149.9 ppm is equivalent to 149.9 µg/mL.[16] This study demonstrates that the extract of *Caulerpa racemosa* exhibits a moderate level ofanticancer activity, as evidenced by the IC_{50} values of 313.2 ppm and 149.9 ppm for the Southeast and North Minahasa regions, respectively.

Caulerpa racemosa has bioactive substances, including alkaloids, phenolics, and flavonoids, that possess promising anticancer properties. Alkaloids are a class of secondary metabolites characterised by their basic nature and the presence of one or more nitrogen atoms inside a cyclic structure. These compounds often have pharmacological effects in both humans and animals. They are typically found in solid (crystalline) form, have a bitter taste optical rotation,and can exist as salt forms soluble in water. They can also be dissolved in organic solvents as free bases or salts.

Moreover, phenolics possess multiple hydroxyl groups connected to an aromatic ring. These chemicals can inhibit the development of coronary heart disease, cancer, and premature ageing.[17] Flavonoids are secondary metabolites with a core structure consisting of two aromatic rings joined by three carbon atoms, typically with oxygen atoms forming heterocyclic connections. These compounds can be categorised as polyphenolic compounds because they possess two or more hydroxyl groups. As a result, they exhibit a relatively high level of acidityand are soluble in polar solvents such as methanol, ethanol, butanol, and ethyl acetate.[18] Thesechemicals have been identified as anti-breast cancer (MCF-7) agents because they hinder cell division mechanisms and trigger apoptosis pathways.

Table 2: Column Chromatography Separation Results

Stationary Phase	Mobile Phase Methanol: Water	Fraction %	Gram
45 grams ODS and 20 grams	100 ml + 400 mL	20	2.50
of crude *Caulerparacemosa*	200 ml + 300 mL	40	2.14
extract	400 ml + 100 mL	80	2.54
	500 mL	10	3.15
		0	

In the antibacterial screening, the susceptibility of the organisms to *Caulerpa racemosa* extracts is presented in Table 1. Column fractions of the plant extracts were separated using the C18 stationary phase, composed of octadecyl silicate, as shown in Table 2. The table shows that shorter alkyl chains in the stationary phase are appropriate for polar and nonpolar molecules.[19] The antibacterial test results indicate that various fractions exhibited inhibition zones ranging from 11.5 mm to 12.9 mm at concentrations ranging from 200 µg/disc to 1000 µg/disc. The positive control agent showed an inhibition zone of 13.4 mm, while the negative controls showed no inhibition.The results of the antibacterial activity test on the 80% fraction of *Caulerpa racemosa* indicate that every extract concentration exhibits antibacterial properties. Similarly, the antibacterial activity of *Caulerpa racemose* against *Mycobacterium smegmatis* is reported in Figure 6. The antibacterial activity of the fractions was concentration-dependent since higher concentrations result in wider zones of inhibition. A concentration of 100 µg/mL, resulting in an average inhibition zone of 11.5 mm, is classified as a strong effect. An inhibitory zone of 13.5 mm was observed at a concentrationof 1000 µg/mL, whereas the positive control exhibits a strong zone of 13.4 mm.[20]

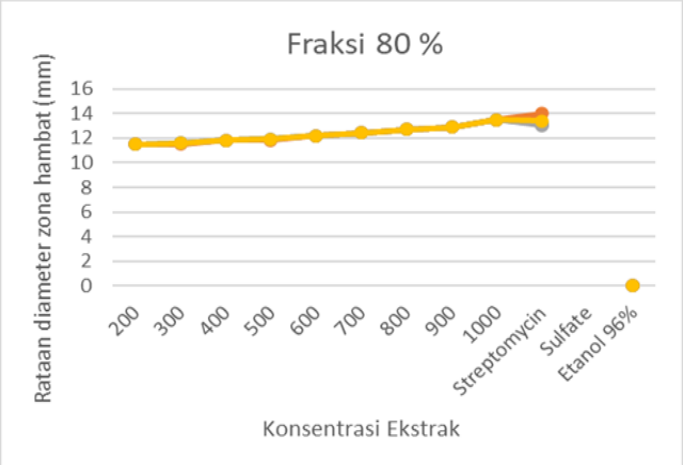

Figure 6: Antibacterial Activity Test Graph on *Mycobacterium smegmatis* using the 80%fraction

Conclusion

In conclusion, the study showed that the crude extracts and fractions of *Caulerpa racemosa* possess antibacterial and anti-breast cancer properties. The compound composition remained consistent across several environments, including areas close to pollution sources or generally free from pollution. The findings demonstrated that the extract derived from *Caulerpa racemosa* from the North and Southeast Minahasa regions exhibited potent anti-breast cancer and antibacterial properties.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that anyliability for claims relating to the content of this article will be borne by them.

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