Anticancer Activity of Marine Macroalgae *Halimeda tuna* from Aceh Waters against Cervical Cancer Cells

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ABSTRACT

Cancer, as a chronic illness, is characterized by a high mortality rate. Current cancer treatments, such as chemotherapy, have significant drawbacks, including long half-lives and numerous adverse effects. Medications derived from natural products offer a safer alternative as supplementary treatments. One such natural product is marine macroalgae, commonly known as seaweed. Secondary metabolites from marine macroalgae exhibit a range of potential biological activities, including anticancer properties. The objective of this study was to determine whether the methanolic extract of *Halimeda tuna* from the Acehnese coast possesses potential anticancer properties. *Halimeda tuna* was extracted using the cold maceration process, which involved immersion in methanol solvent for 24 h. The MTT assay was used to assess the cytotoxicity of crude *H. tuna* extract against HeLa cervical cancer cells. GC-MS testing and phytochemical analysis were conducted to identify the compounds present in the extract. The yield of *H. tuna* extract was 0.168±0.025%. The MTT assay determined the IC_{50} value of *H. tuna* against HeLa cells to be 126.460±46.167 $\mu g \cdot mL^{-1}$. Phytochemical analysis revealed the presence of flavonoids, steroids, and alkaloids, while GC-MS analysis identified fatty acids, steroids, and flavonoids in the extract. These compounds may contribute to the anticancer effects observed, consistent with an IC_{50} value that indicates moderate cytotoxicity. Further research is required to explore the potential of *H. tuna* extract as an alternative anticancer agent.

Keywords: Anticancer, Cytotoxic, HeLa, MTT, Seaweed

INTRODUCTION

Cancer is a metabolic disease and one of the leading causes of death and morbidity worldwide. The development of cancer is driven by a combination of genetic, epigenetic, environmental, and hormonal factors that lead to cellular mutations (Siegel *et al*., 2016). While chemotherapy remains one of the most effective treatments for cancer, it is often accompanied by significant adverse effects, including nausea, diarrhea, pain, hair loss, and fatigue. These side effects can severely impact a patient's quality of life and may increase the risk of mortality (Warrington *et al*., 2019).

In many parts of Asia, particularly among populations with strong traditional medicine practices, there is a widespread use of herbal products, dietary supplements, and other complementary therapies such as massage (Salleh *et al*., 2021). Seaweed, or

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macroalgae, is one such natural ingredient that has gained attention for its potential therapeutic properties. Seaweeds are rich in secondary metabolites, including flavonoids, phenolics, and tannins, which exhibit various bioactive properties, including anticancer effects (Ranahewa *et al*., 2019).

Several studies have highlighted the anticancer potential of different species of seaweed. For example, *Sargassum hystrix* has shown promise in anticancer activity (Husni *et al*., 2021; Gazali *et al*., 2022a), as have *Padina pavonica* (Arunkumar *et al*., 2021), *Kappaphycus alvarezii* (Papitha *et al*., 2020), and *Halimeda macroloba* (Husni *et al*., 2024). Notably, *Halimeda tuna*, a green marine macroalga from the waters of Aceh, has been reported to possess potential anticancer properties against lung cancer cells (Husni *et al*., 2024). However, its efficacy against HeLa cervical cancer cells has not yet been investigated.

The bioactivity of marine macroalgae from Aceh's coastal regions has been previously studied. Species such as *Halimeda opuntia* (Gazali *et al*., 2019a), *Chaetomorpha antennina* (Gazali *et al*., 2020), *Halimeda macroloba* (Gazali *et al*., 2019b), *Enteromorpha flexuosa* (Gazali *et al*., 2021), *Caulerpa racemosa* (Gazali *et al*., 2022b), and *Boergesenia forbesi* (Gazali *et al*., 2023) have demonstrated various biological activities. Given the promising results of previous studies, this research aims to investigate the anticancer activity of *H. tuna* extract specifically against HeLa cervical cancer cells, potentially expanding the therapeutic applications of this marine macroalga.

MATERIALS AND METHODS

Samples collection and identification

The *Halimeda tuna* samples were collected from the waters of Aceh on April 15, 2021, and identified at the Laboratory of Plant Systematics, Faculty of Biology, Gadjah Mada University. The fresh seaweed samples were air-dried, cleaned of any adhering dirt, and placed in plastic containers to prevent contamination. They were then stored in a freezer until extraction. Before extraction, the seaweed was cut into small pieces, but not crushed, to facilitate filtering.

Extraction and sample preparation

The extraction of *H. tuna* was carried out using a modified cold maceration method, as described by Yang *et al*. (2011). A total of 250 g of *H. tuna* powder was immersed in 2.5 L of methanol solvent for 24 h at room temperature. The resulting filtrate was filtered using Whatman Paper No. 42. The solvent was then separated using a vacuum rotary evaporator and nitrogen evaporation. Finally, the extracted paste was freeze-dried to increase its concentration, following the method described by Ibrahim and Kebede (2020).

HeLa cell preparation

HeLa cell lines were cultured under atmospheric conditions in a 5% CO_2 incubator at 37 °C using RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S), and 1% L-glutamine (Audus *et al*., 2002). After being washed with phosphate-buffered saline (PBS), the cells were detached using trypsin-EDTA. The cell count was determined using a hemocytometer with trypan blue staining. The cells were resuspended to a density of 5×10^4 cells·mL^{-1} and cultured in a 96-well microplate for 24 h under the same conditions prior to treatment (Hopp *et al*., 2017).

MTT anticancer activity test

HeLa cells were seeded in 96-well flat-bottom plates (SPL Life Sciences) at a density of 10^4 cells per well in 95 µL of complete RPMI 1640 medium. The cells were incubated in a 5% CO_2 atmosphere at 37 °C for 24 h to allow cell adhesion. Seven compounds were dissolved in sterile dimethyl sulfoxide (DMSO) to create seven different concentrations: 20, 50, 100, 200, 500, 1,000, and 2,000 µM for each compound. Following the addition of the compounds, the cells were incubated at 37 °C in a 5% CO_2 atmosphere for 24, 48, and 72 h to assess the cytotoxicity of the compounds. MTT (Sigma) was dissolved in PBS (phosphate-buffered saline) at a concentration of

5 mg·mL^{-1} and filtered. Each well received 10 µL of MTT solution, and the cells were incubated for 3.5 h. After incubation, the cells were centrifuged at 1,800 rpm for 10 min. The supernatant was removed, and 100 µL of DMSO was added to each well. The plates were shaken for 15 min to dissolve the formazan crystals completely. Absorbance was measured at 540 nm (Omar *et al*., 2018).

GC-MS analysis

The sample was analyzed using a gas chromatography-mass spectrometry (GC-MS) system with a capillary column under a temperature range of 70 °C to 325 °C in splitless mode. The gas flow rate was optimized after adjusting the column to a concentration of 25 to 30 µg·mL^{-1}. The sample was methylated with methanol and boron trifluoride before injection. The injected volume depended on the type of detector used. The column utilized was a GC Capillary Column RTX-5MS with a length of 30 m, a film thickness of 0.25 µm, and a column diameter of 0.25 mm ID. The stationary phase was HP-5MS 5% phenyl methyl siloxane (Diningrat *et al*., 2021). During elution, the detector signal was recorded as a chromatogram. The chromatogram was processed using a microprocessor to identify each component, which was compared to a library database based on the similarity index (SI) (Lusiantika *et al*., 2019).

Phytochemical analysis

Phytochemical screening was performed using solubility tests, colorimetric tests, or color reactions with characteristic reagents and precipitation reactions. Different reagents were used to test each group of phytochemical compounds (Hayat *et al*., 2020).

Flavonoid test (Ben et al., 2013)

A 0.005 g sample was boiled in 2.5 mL of 70% ethanol and filtered. A drop of 10% NaOH was added to the filtrate. A yellow or orange color indicated the presence of flavonoids.

Steroid/triterpenoid test (Ben et al., 2013)

A 0.005 g sample was treated with ten drops of chloroform and dried on a drip plate, then homogenized with five drops of acetic acid (CH_3COOH) and two drops of 96% sulfuric acid (H_2SO_4). A blue/green color indicated the presence of steroids, while a red/purple color indicated triterpenoids.

Saponin test (Ben et al., 2013)

A 0.005 g sample was dissolved in 2.5 mL of hot distilled water, shaken until frothy, and then cooled for ten minutes. Persistent foam after adding one drop of 2 N HCl indicated the presence of saponins.

Tannin test (Widowati et al., 2021)

A 0.005 g sample was dissolved in 2.5 mL of hot distilled water and filtered. Three drops of 1% $FeCl_3$ were added to the filtrate. A dark green color indicated the presence of tannins.

Alkaloid test (Widowati et al., 2021)

A 0.005 g sample was treated with five drops of NH_3 and filtered. The filtrate was then treated with five drops of 2 M H_2SO_4 and allowed to stand until a separate layer formed. The first layer was divided into three portions, each treated with Dragendorff's, Mayer's, or Wagner's reagents. An orange precipitate with Dragendorff's, a white precipitate with Mayer's, and a brown precipitate with Wagner's reagents indicated the presence of alkaloids.

Hydroquinone phenol test (Manongko et al., 2020)

A 0.005 g sample was dissolved in 2 mL of 70% ethanol and treated with two drops of $FeCl_3$. The mixture was homogenized and observed for a color change. A bluish-green color indicated the presence of phenol hydroquinone.

Statistical analysis

All data were presented as the mean ± standard deviation (SD) of three independent trials (triplicates). Post-host test with a one-way analysis of variance (ANOVA) was performed to evaluate statistical significance at a confidence level of $p<0.05$. SPSS software was used for data analysis. Cytotoxic test results from the MTT assay were used to calculate the IC_{50} value, representing the concentration required to inhibit 50% of cancer cell proliferation. Absorbance values were processed by generating a dose-response curve, plotting log concentration (log C) against the probit value for the percentage of cell mortality, to determine the IC_{50} value.

RESULTS AND DISCUSSION

Yield of extract

The yield percentage of the crude extract of *Halimeda tuna* was determined by comparing the weight of the obtained paste to the initial weight of the wet base sample (250 g).

The crude extract yield of *H. tuna* in this study averaged 0.168±0.025%, aligning closely with the findings of Muzaki *et al*. (2018), who reported a methanol extract yield of 0.34% for *H. macroloba*, likely due to the similar use of wet samples. Conversely, Milović *et al*. (2019) observed a significantly higher yield of 3.75±0.5% for *H. tuna* using dichloromethane as the solvent. This variation can be attributed to various factors such as solvent type, sample preparation, genetic variability, and environmental conditions, all of which influence the quality and yield of herbal extracts. Additionally, the condition of the samples (fresh or dried) and their storage duration can further impact extract yield and quality (Kunle and Egharevba, 2013).

Cytotoxicity of Halimeda tuna extract

The anticancer potential of the *H. tuna* extract was evaluated using the MTT assay against HeLa cancer cells. Results demonstrated dose-dependent inhibition of HeLa cell proliferation (Table 1), with an IC_{50} value of 126.460±46.167 $\mu g \cdot mL^{-1}$ (Table 2), classifying the extract's cytotoxicity as moderate ($100\ \mu g \cdot mL^{-1} < IC_{50} < 1{,}000\ \mu g \cdot mL^{-1}$). Comparatively, the positive control, Doxorubicin, displayed significantly higher efficacy, with an IC_{50} of 7.647±2.575 $\mu g \cdot mL^{-1}$.

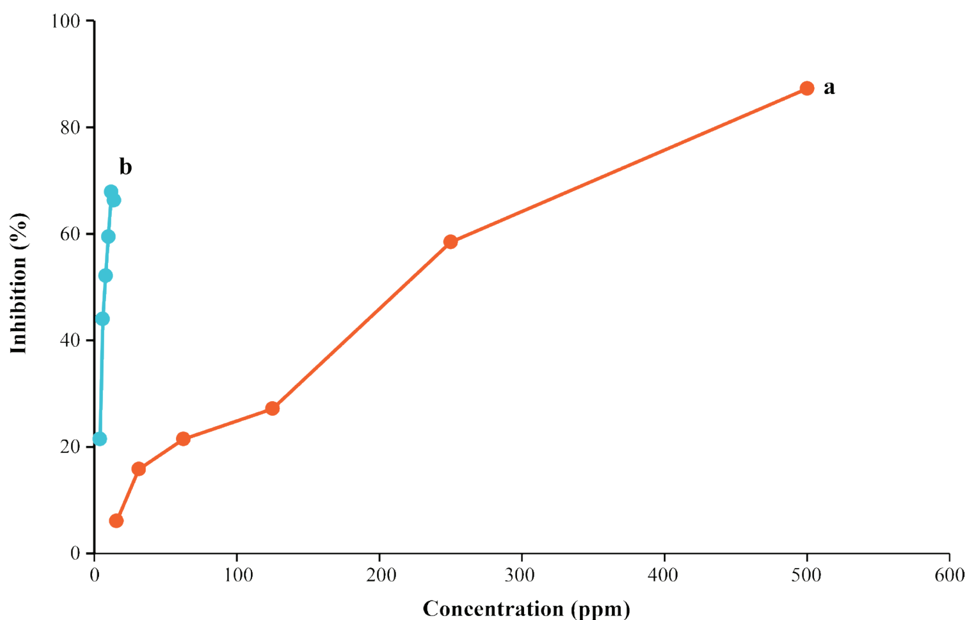

Figure 1. Inhibition activity of *Halimeda tuna* extracts (a) and doxorubicin (b) at various concentrations on HeLa cell proliferation.

Table 1. IC_{50} values of *Halimeda tuna* extract compared to Doxorubicin against HeLa cells.

Sample	Sample code	IC_{50} ($\mu g \cdot mL^{-1}$)
Halimeda tuna	HT	126.460 ± 46.167^{a}
Doxorubicin	Dox	7.647 ± 2.575^{b}

Note: Different superscript letters indicate significant difference ($p<0.05$).

Table 2. Volatile compounds in *Halimeda tuna* extract: Retention time, area (%), component, group, activity, and similarity index of identified peaks.

Peak	Retention time	Area (%)	Component	Group	Activity	Similarity index
1	12.308	32.34	Stigmasta-5,22-dien-3-ol	Steroid	Antioxidant, antimycobacterial (tuberculosis), anticancer, inhibition of chemocarcinogen	19
2	13.572	7.73	Androst-4-ene-3,17-dione	Steroid	Osteoporosis, antiinfectives, hyperglycemia (antidiabetic)	21
3	18.010	5.03	1-Docosanol	Steroid	Antiviral	57
4	19.683	0.01	1-Hexadecanol	Fatty alcohol	Antioxidant, antimicrobial	61
5	20.441	27.41	14-Beta-H-Pregna	Steroid	Cancer prevention	63
6	20.883	6.78	Dodecanoic acid/ Lauric acid	Fatty acid	Antimicrobial, relieve neuro-inflammatory	34
7	21.065	20.73	Hexadecanoic acid	Fatty acid	Anti-inflammatory, antiviral, antioxidant	70

Previous studies have reported the anticancer activity of various seaweed species against HeLa cells, showing differing levels of effectiveness. For instance, *Halimeda* sp. extracted with a dichloromethane (1:1) solvent yielded an IC_{50} of 17.92 ± 1.54 $\mu g \cdot mL^{-1}$ (Milović *et al.*, 2019), while *H. macroloba* exhibited a higher IC_{50} of 137.38 $\mu g \cdot mL^{-1}$ (Sanger *et al.*, 2021). Among brown seaweeds, the methanol extract of *Dictyota dichotoma* showed an IC_{50} of 17.20 ± 0.90 $\mu g \cdot mL^{-1}$ (El-Shaibany *et al.*, 2020), and *Padina pavonica* displayed a notable IC_{50} of 10 $\mu g \cdot mL^{-1}$ (Arunkumar *et al.*, 2021). Red seaweed methanol extracts have exhibited even greater cytotoxicity, with *Kappaphycus alvarezii* showing an IC_{50} of 10.2 $\mu g \cdot mL^{-1}$ (Papitha *et al.*, 2020) and Jania rubens recording an IC_{50} of 37.9 $\mu g \cdot mL^{-1}$ (El-Shafay *et al.*, 2022).

Several factors may influence the anticancer activity of *H. tuna* in this study, including seaweed characteristics, extraction methods, extract properties, cancer cell type, and dosage (El-Beltagi *et al.*, 2022). Environmental factors such as sunlight, temperature, and nutrient availability affect the production of bioactive compounds (Michalak *et al.*, 2022). The

extraction process is critical, as different methods yield varying compositions and potencies, with some being more effective for isolating anticancer compounds (Irianto *et al*., 2024). Purification can enhance activity by removing impurities or concentrating bioactive compounds, while compound stability impacts effectiveness (Mensah *et al*., 2023). Additionally, the extract's potency may vary with the targeted cancer cell type and dosage applied.

HeLa cell cytomorphology

Morphological changes in HeLa cells before and after treatment were observed under an inverted microscope. Healthy HeLa cells exhibited polygonal shapes with closely packed colonies and microvilli (Suganya *et al*., 2020). Post-treatment with Doxorubicin, cells displayed significant structural damage, including membrane blistering, cell detachment, and colony disintegration. Similarly, *H. tuna* extract-treated cells showed shrinkage and reduced colony integrity, albeit to a lesser extent (Figure 2).

GC-MS test analysis

The GC-MS analysis of *H. tuna* extract identified seven peaks, indicating a mixture of bioactive compounds (Figure 3). Key compounds included flavonoid (flemichapparin A) (NCBI, 2022), steroids [Stigmasta, Androst-4-ene-3,17-dione, Estra-1,3,5(10)-trien-17-one, 5 alpha-Androstan-17-one, and 1-Docosanol (Baranovsky and Litvinovskaya, 2019)], and fatty acids [lauric acid, 4-hydroxynoic acid, dodecanoic acid, hexadecanoic acid, and octadecanoic acid (Utami *et al*., 2018)]. The retention time, molecular weight, and similarity index were matched against library databases.

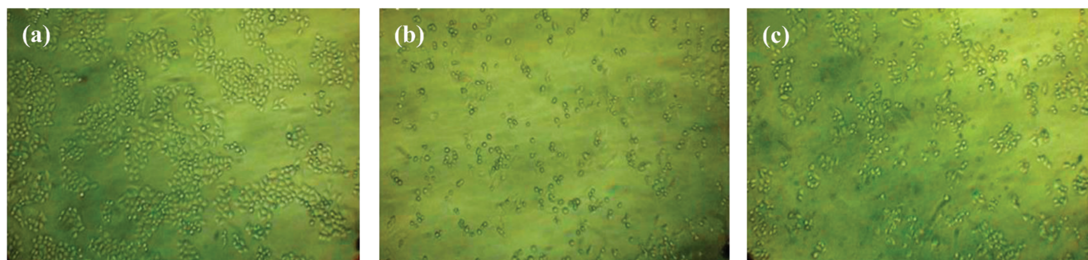

Figure 2. Cytomorphology of HeLa cells: (a) negative control (untreated HeLa cells), (b) HeLa cells exposed to 8 g·mL^{-1} Doxorubicin, and (c) HeLa cells exposed to 250 µg·mL^{-1} *Halimeda tuna* extract.

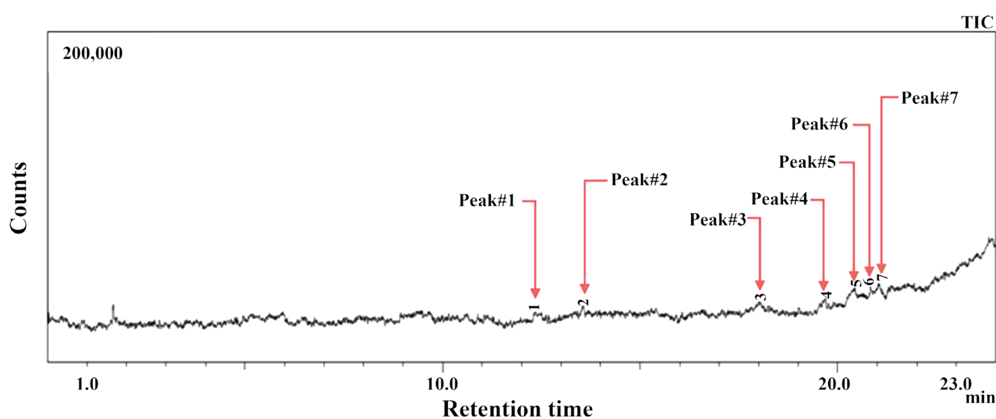

Figure 3. Chromatogram of *Halimeda tuna* extract showing the separation of volatile compounds, with peaks representing individual compounds at varying retention times and areas, reflecting their relative abundance and potential bioactivity.

The findings align with known biological activities, supporting the therapeutic potential of these compounds (Utami *et al*., 2018; Baranovsky and Litvinovskaya, 2019). A detailed list of compounds and their activities is provided in Table 3.

Table 3 highlights several volatile compounds with bioactivity linked to cancer prevention. Peaks 1, 5, and 7, representing 32.34%, 27.41%, and 20.73% of the total area, were identified as major compounds. The compound with the largest area and a similarity index of 70 was selected for further analysis.

Stigmasta-5,22-dien-3-ol (peak 1), a stigmasteroid, the is known for its antioxidant, antimycobacterial (anti-tuberculosis), anti-inflammatory, anticancer, and chemocarcinogen-suppressing properties (Loori *et al*., 2021). This compound is prevalent in *Halimeda* species, such as *H. opuntia*, where it constitutes 54.74% of the total composition (Nasrudin *et al*., 2017). However, low SI (19) for Stigmasta in this study suggests a minor role in anticancer activity.

14-Beta-H-Pregna (peak 5), with an SI of 63 exhibits anticancer and antidiabetic effects (Zhang *et al*., 2012). This steroid was also found in microalgae *Chlorella vulgaris* (55% area) (Kusumaningrum and Zainuri, 2018) and the medicinal herb *Verbascum pseudoholotricum* (98% SI), which has antibacterial, anti-inflammatory, and antioxidant properties (Yabalak *et al*., 2020). Fatty acids (peak 7), including hexadecanoic acid, octadecanoic acid, dodecanoic acid, and octadecane, are known for their antibacterial, antiviral, antioxidant, and anti-inflammatory properties (NCBI, 2022). These compounds reduce reactive oxygen species (ROS), mitigating ROS-induced diseases such as cancer (Kim *et al*., 2010).

We have reported that GC-MS analysis of *H. opuntia* and *H. macroloba* confirmed hexadecanoic acid as a key component with cytotoxic effects on the HCT-116 colorectal cancer cells (Nazarudin *et al*., 2020). In this study, the retention time (RT) of hexadecanoic acid in *H. tuna* (21.065) closely matches prior findings for *H. macroloba*, supporting its role as a potential bioactive compound.

Phytochemical analysis

Phytochemical analysis of the methanol extract of *H. tuna* revealed the presence of steroids, flavonoids, and alkaloids (Table 3), consistent with prior studies identifying these as the primary secondary metabolites in *H. tuna* extract. Concentrations were reported as high for steroids, medium for flavonoids, and low for alkaloids.

Table 3. Phytochemical analysis results of *Halimeda tuna* extract and corresponding indicators.

Phytochemical	Result	Indicator
Flavonoid	++	Yellow/orange color
Steroid	+++	Blue-green color
Triterpenoid	-	Red-purple color
Saponin	-	Foam
Alkaloid		
- Dragendorff	+	Orange precipitate
- Meyer	-	White precipitate
- Wagner	-	Brown precipitate
Tannin	-	Blue-black color
Phenol hydroquinone	-	Orange/red color

Note: + = Low; ++ = Moderate; +++ = High

Flavonoids, a class of polyphenols, play a protective role in plant against UV radiation, pests, and microbial attacks. Studies on humans, animals, and cell cultures suggest that flavonoids offer significant health benefits particularly their anticancer potential. These compounds induce apoptosis by mechanisms involving redox regulation, DNA damage, and protein kinase activity, thereby inhibiting cancer cell proliferation, suppressing angiogenesis and metastasis, halting the cell cycle, and promoting both apoptosis and autophagy (Kopustinskiene *et al*., 2020). Apoptosis-inducing capabilities of flavonoids suggest that *H. tuna* extracts may hold promise as a anticancer agent.

Steroids, a class of terpenoid lipids with a four-ring carbon skeletons, exhibit diverse biological activities, including cholesterol-lowering and anticancer properties. Their anticancer effects are partly attributed to their antiangiogenic activity. For example, stigmasterol, a well-known steroid, has shown potential in preventing cancers such as ovarian, breast, prostate, and colon cancer due to its antioxidant, hypoglycemic, and thyroid- inhibitory properties (Nasrudin *et al*., 2017).

Alkaloids, nitrogenous base compounds, induce apoptosis by disrupting peptidoglycan synthesis, a critical component of cell walls, leading to cell death. Alkaloids' basic groups interact with DNA and cell wall amino acids, disrupting genetic stability and cellular metabolism, which can result in cell lysis or destruction (Robinson, 2005).

CONCLUSIONS

The methanol extract of *Halimeda tuna* from Aceh showed moderate cytotoxicity against HeLa cells, with an IC_{50} value of 126.460±46.167 $\mu g \cdot mL^{-1}$, primarily attributed to steroids and fatty acids. While promising, its effectiveness is lower than established agents like Doxorubicin. Future studies should optimize extraction methods, isolate and characterize active compounds, and conduct *in vivo* experiments. Research should explore synergistic effects with other compounds, test against diverse cancer cell lines, and investigate underlying mechanisms of to fully understand therapeutic potential of *H. tuna* and its potential contribution to novel cancer treatments.

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