ACCEPTED MANUSCRIPT • **OPEN ACCESS**

Cytotoxic Activity of Green Seaweed *Halimeda tuna* Methanolic Extract Against Lung Cancer Cells

To cite this article before publication: A. Husni, M. Gazali, N. Nurjanah, R. Syafitri, A. Matin, and Z. Zuriat. (2023). *J. Multidiscip. Appl. Nat. Sci.* in press. https://doi.org/10.47352/jmans.2774-3047.172.

Cytotoxic Activity of Green Seaweed *Halimeda tuna* Methanolic Extract Against Lung Cancer Cells

Amir Husni[1,a)]; Mohamad Gazali[2,6b)]; Nurjanah Nurjanah[3,c)]; Rina Syafitri[4,d)], Abdul Matin[5,6e)]; Zuriat Zuriat[7,f)]

[1]*Department of Fisheries, Universitas Gadjah Mada, Yogyakarta-55281 (Indonesia)*
[2]*Department of Marine Science, Teuku Umar University, West Aceh-23681 (Indonesia)*
[3]*Department of Aquatic Product Technology, IPB University, Bogor-16680 (Indonesia)*
[4]*Department of Agribusiness, Teuku Umar University, West Aceh-23681 (Indonesia)*
[5]*Department of Food Processing and Engineering, Chattogram Veterinary and Animal Sciences University, Chattogram-4225 (Bangladesh)*
[6]*Institute of Marine Biotechnology, Universiti Malaysia Terengganu, Kuala Terengganu-21030 (Malaysia)*
[7]*Department of Fisheries, Teuku Umar University, West Aceh-23681 (Indonesia)*

[a)]a-husni@ugm.ac.id
[b)]Correspondence: mohamadgazali@utu.ac.id
[c)]nurjanah@apps.ipb.ac.id
[d)]rinasyafitri@utu.ac.id
[e)]abmatin@cvasu.ac.bd
[f)]zuriat@utu.ac.id

ORCIDs:
First AUTHOR : http://orcid.org/0000-0003-3327-9848
Second AUTHOR : https://orcid.org/0000-0001-7575-3582
Third AUTHOR : https://orcid.org/0000-0001-7925-1782
Fourth AUTHOR : https://orcid.org/0000-0002-2334-4397
Fifth AUTHOR : https://orcid.org/0000-0003-3029-4989
Sixth AUTHOR : https://orcid.org/0009-0000-6296-1788

ACKNOWLEDGEMENT

This research supported by the Ministry of Education and Culture Republic Indonesia through DRTPM Dikti Grant for the Inter-University Cooperation Research Scheme (PKPT) with Contract Number 037/UN59.7/PG.02.00.PT/2022.

AUTHOR CONTIBUTIONS

Conceptualization, A.M. and M.G.; Methodology, A.M and M.G; Software, A.M.; Validation, A.H., M.G. and A.M.; Formal Analysis, A.M. and M.G.; Investigation, A.H.; Resources, M.G.; Data Curation, M.G.; Writing–Original Draft Preparation, M.G.; Writing – Review & Editing, A.M. M.G. A.M. and N.N., R.S., and Z.Z.; Visualization, .X.; Supervision, A.M. and N.; Project Administration, M.G.; Funding Acquisition, M.G.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

Cytotoxic Activity of Green Seaweed *Halimeda tuna* Methanolic Extract Against Lung Cancer Cells

Abstract. Lung cancer is a malignant tumor that attacks the lungs generated by carcinogenic free radicals such as cigarette smoke. Seaweed contains bioactive compounds that have the potential to reduce cancer-causing free radicals. This study aimed to determine the phytochemical content and cytotoxic activity of *Halimeda tuna* seaweed extract against lung cancer cells (A549). The *H. tuna* sample was macerated using methanol for 24 h. Cytotoxic test of *H. tuna* crude extract used the MTT test against A549. The crude extract was phytochemically tested and analyzed using gas chromatography–mass spectrometry (GC-MS). The results showed that the *H. tuna* crude extract had cytotoxic activity against A549 with an IC_{50} value of 2771 µg/mL. The phytochemical test showed that *H. tuna* crude extract contained flavonoids and steroids. GC-MS spectra showed the presence of fatty acid compounds including palmitic acid, oleic acid, myristic acid, palmitoleic acid and stearic acid. Based on the results can be concluded that *H. tuna* extract had cytotoxic activity against A549 with low cytotoxicity to be used as a chemo-preventive agent.

Keywords: anticancer; cytomorphology; flavonoid; green seaweed; steroid

1. INTRODUCTION

Lung cancer is one of the most dangerous deadly diseases. The death rate from lung cancer worldwide can reach one million people annually; even in Indonesia, this disease is ranked 4th in the world [1]. Cancer is a metabolic syndrome that is one of the leading causes of death and morbidity worldwide. Primary cancer-triggering factors include genetic, epigenetic, environmental, and hormonal that cause mutations [2]. The leading cause of lung cancer is caused by long-term exposure to carcinogenic substances, especially substances that enter through the respiratory process, such as air pollution and cigarette smoke. Many have reported that lung cancer is associated with smoking habits. As many as 65% of the risk of lung cancer is suffered by males, especially those aged over 40 years [1]. The most effective cancer treatment, namely chemotherapy, still has various side effects, such as nausea, hair loss, pain, fatigue, and diarrhea. In the long term, these symptoms can harm the patient's quality of life and are at risk of death [3].

Most Asian people use complementary medicine such as dietary supplements, herbal products, and other traditional treatments [4]. One of the herbal medicines or natural ingredients from the fisheries sector is seaweed. Seaweed contains various secondary metabolites, such as flavonoids, phenolics, and tannins [5]. Seaweed also contains phenolic compounds, polysaccharides, polyunsaturated fatty acids (PUFAs), proteins, vitamins, and minerals. These compounds show biological activity and have the potential to be used as drugs to ward off cancer, tumors, thrombosis, diabetes, inflammation, and other degenerative diseases [5-8]. These bioactive compounds can be used as antioxidant, anticancer, antibacterial, anti-inflammatory, and antiviral agents [9].

Several studies have shown that seaweed from the Halimeda genus consists of bioactive compounds, including polyphenols, diterpenes, fatty acids, and sterols, that show anticancer activities [10,11]. One potential seaweed species as an anticancer is the green seaweed *Halimeda tuna* from Aceh waters. Previous research has been carried out related to the bioactivity of seaweed originating from Aceh waters, such as *H. macroloba* [12], *H. opuntia* [13], and *H. tuna* [14]. Green seaweed is abundant in Indonesia and mainly used in the food sector, however, green seaweed is rarely used in the pharmaceutical and health fields. Research shows that green seaweed contains bioactive compounds such as alkaloids, flavonoids, tannins, saponins, and steroids [15]. Some of these bioactive compounds can potentially reduce free radicals that cause cancer. Several studies have been conducted on the cytotoxic activity of green seaweed, namely *Boergesenia forbesii*, which has high cytotoxic activity so it has the potential to become an anticancer [16]. Puc et al. [17] reported that *H. tuna* has cytotoxic activity against cervical cancer cells (HeLa), laryngeal cancer cells (Hep-2), and nasopharyngeal cancer cells (KB). Several species of *Halimeda sp.* contain halimedatrial compounds (diterpenetrialdehyde), which have cytotoxic activity [18], so they have the potential as anticancer. However, the content of seaweed bioactive compounds can vary depending on the type of species, age of harvest, and environmental conditions of the habitat or place of growth [19]. Therefore, this study aimed to determine the anticancer activity of green seaweed *H. tuna* methanol extract against lung cancer cells (A549).

2. MATERIALS AND METHODS

2.1. Materials. The materials used in this study were green seaweed *H. tuna*, methanol (Sigma Aldrich,), ethanol, NaOH, chloroform, anhydrous acetic acid, HCl, $FeCl_3$, NH_3, $CHCl_3$, H_2SO_4, Dragendorfs reagent, Meyer's reagent, Wagner's reagent, lung cancer cells (A549)

(BPPT, Tangerang), RPMI medium, Fetal Bovine Serum (FBS), streptomycin penicillin, doxorubicin, fugizone, formazan, MTT, SDS. The tools used in this study included laboratory glasswares, Whatman filter paper no.42, rotary evaporator (DLab RE100-Pro, Germany), nitrogen gas evaporator, hot plate stirrer (F20500011 Velp AREC Heating stirrer, Italy), ELISA microplate reader (Heales MB-580), 96-well microplate, and CO_2 incubator (Memmert ICO150Med, Germany).

2.2. Methods

2.2.1. Preparation and Identification of Samples. Samples of green seaweed *H. tuna* were collected from the coast of Lhok Bubon, Samatiga Subdistrict, West Aceh District, Aceh Province. The samples were washed with fresh water to remove the adhering sand and dirt. The wet samples were then dried at room temperature. The wet and dry samples were sent to Universitas Gadjah Mada, Yogyakarta. Fresh seaweed samples were identified at the Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada, to determine the specific type, whereas, the dry samples were cut into 1 cm pieces using scissors. The seaweed was weighed and stored at -20 °C.

2.2.2. Extraction of Seaweed. The extraction of *H. tuna* was carried out according to Yang et al. [24] with modifications. Samples of dried *H. tuna* were weighed as much as 250 g. The sample was macerated with 2 L of methanol for 24 h at room temperature and then the filtrate was filtered to remove the remaining residue carried. The filtrate was evaporated using a rotary evaporator at a temperature of 40 °C at 60 rpm. The sample was further treated using nitrogen gas to produce an extract in the form of a more concentrated paste and then extracted in the freeze dryer.

2.2.3. Anticancer Activity Test. An anticancer activity test was conducted to determine whether the extracted sample had the potential as an anticancer of the lungs. The anticancer activity test was carried out based on the method according to Husni et al. [20]. Anticancer activity tests included an A549 culture, cytotoxicity, and cytomorphological testing. A549 cancer cells were cultured in RPMI medium, then added 10% FBS, streptomycin, penicillin, and fungizone. Then the mixture was incubated with 5% CO_2 at 37°C to obtain an A549 cell culture. Furthermore, the cytotoxicity test was carried out using the MTT method. A549 cells were placed on a 96-well culture microplate that included cancer cell treatment with samples,

positive controls with doxorubicin, and negative controls without sample treatment. Then the mixture was incubated with 5% CO_2 at 37 °C for 24 h. After that, the media was discarded and then it was mixed with 100 µL MTT and incubated again for 4 h. After that, the purple format was dissolved in 100 µL 10% SDS and allowed to stand for 12 h at room temperature. Cell growth was read using an ELISA microplate reader at a wavelength of 570 nm. The percentage of live cells after exposure to fucoidan was calculated using the following equation 1.

$$\%\ Life\ cell = \frac{absorbance\ of\ treatment - absorbance\ of\ medium}{absorbance\ of\ cell\ control - absorbance\ \ of\ medium} \times 100\% \quad \textbf{(1)}$$

2.2.4. Phytochemical assay

2.2.4.1. Flavonoid. The flavonoid test was carried out to determine the content of flavonoid compounds in the sample. Five mL of 70% ethanol was added to 0.05 g of the extracted sample, then heated and filtered. Then the filtrate was taken, and two drops of 10% NaOH were added. If the color changes to yellow or orange, the sample contains flavonoids.

2.2.4.2 Saponin. The saponin test was carried out based on the method as described by Lubis et al. [21]. The saponin test was carried out to determine the content of saponin compounds in the sample. A total of 0.05 g of the extracted sample was dissolved into 10 mL of hot distilled water and then shaken vigorously until foamy and cooled. Then 1 drop of 2 M HCl was added. If the foam does not disappear, then the sample contains saponins.

2.2.4.3 Steroid and Triterpenoid. Steroid and triterpenoid tests were carried out as follows: chloroform was added to 0.05 g of the extracted sample to the drip plate and then allowed to dry. Then ten drops of anhydrous acetic acid were added and stirred until homogeneous. Then three drops of 96% sulfuric acid were added. If it is blue or green, then the sample contains steroids. If it is red or purple, the sample contains triterpenoids [21].

2.2.4.4 Tannin. The tannin test was carried out based on the method as described by Widowati et al. [22]. A total of 0.1 g of sample was dissolved in 10 mL of hot distilled water and filtered. Then 5 mL of the sample filtrate was added with 3 drops of 1% $FeCl_3$. If the results show a blue-black color, the sample contains tannins.

2.2.5 Gas chromatography-mass spectrometry (GC-MS) analysis. GC-MS analysis was performed to identify the profile of bioactive compounds in *H. tuna* methanolic extract. The GC-MS analysis was carried out based on the method as described by Hidayah [23]. The sample to be analyzed by GC-MS was first dissolved in 5 mL methanol. Then the GC-MS analysis was carried out by injecting the sample into the injection port at a temperature of 290 °C. The volatilized sample was carried by Helium gas at a flow rate of 1 mL/min through the GC column. The initial injection temperature was 80 °C and increased by 10 °C/min with a final temperature of 300 °C. Compounds are detected in the MS system by colliding compounds with electrons to form ionized molecules and record fragmentation patterns [24].

2.3 Statistical Analysis. The percentage data of inhibition was then converted to a linear regression equation calculating the IC_{50} value. The IC_{50} values of the linear regression results of each sample were statistically tested using SOVS (one-way ANOVA) and Tukey HSD test with a 95% confidence level.

3. RESULTS AND DISCUSSIONS

3.1. Yield of Extract. The yield is the result of a comparison between the total mass of *H. tuna* extract in the form of paste with the initial mass of *H. tuna* in the form of dried seaweed [30]. The yield of *H. tuna* methanolic extract obtained was 0.17±0.04%. The methanol extract of H. tuna had a lower yield when compared to the methanol extract of *H. macroloba* (0.34%) and the ethyl acetate extract of *H. macroloba* (0.28%), but higher than the *n*-hexane extract of *H. macroloba* (0.04%) [25]. Gazali et al. [12] also reported that the yield of ethanol extract of *H. macroloba* (2.32%) was higher than the yield of ethyl acetate extract (1.26%), and *n*-hexane extract (1.03%). The difference in yield can be caused by the type of solvent and different species. Different solvents can affect the yield due to the level of polarity. According to Muzaki et al. [30], the yield value decreases along with the decrease in the polarity of the solvent. In addition, the solvent will attract bioactive compounds that have the same polarity. The type of seaweed species also affects the yield because it depends on its compounds. According to Purwaningsih and Deskawati [26], the content of bioactive compounds in seaweed is influenced by the type of species, harvest season, harvest age, and geographical location.

3.2 Anticancer Activity. *H. tuna* methanolic extract was assayed for its cytotoxic activity against A549. The inhibition of the growth of A549 by *H. tuna* methanolic extract and doxorubicin is presented in Figure 1 while their IC_{50} is shown in Table 1. The morphological attributes of the cells were monitored under an inverted microscope after the cells were incubated. The morphological attributes of A549 that were exposed and not exposed to *H. tuna* extract are illustrated in Figure 2. A cytotoxicity test was carried out on *H. tuna* methanolic extract against A549 to determine whether the sample had potential as an anticancer and directly affected cell death [17]. The MTT test is a method that can be used to determine the toxic properties of a compound. The MTT test results of *H. tuna* extract, and doxorubicin on A549 (Figure 1) showed that the dose given to cancer cells was directly proportional to the inhibition of cancer cell growth. *H. tuna* extract with a dose of 500 µg/mL could hinder the growth of cancer cells by 28.72% while doxorubicin (as a standard drug) at a dose of 14 µg/mL could hinder the growth of cancer cells by 39.68% (Figure 1). This is because doxorubicin is a widely used drug for anticancer chemotherapy. However, doxorubicin works non-selectively and is toxic to cancer cells and normal cells [27].

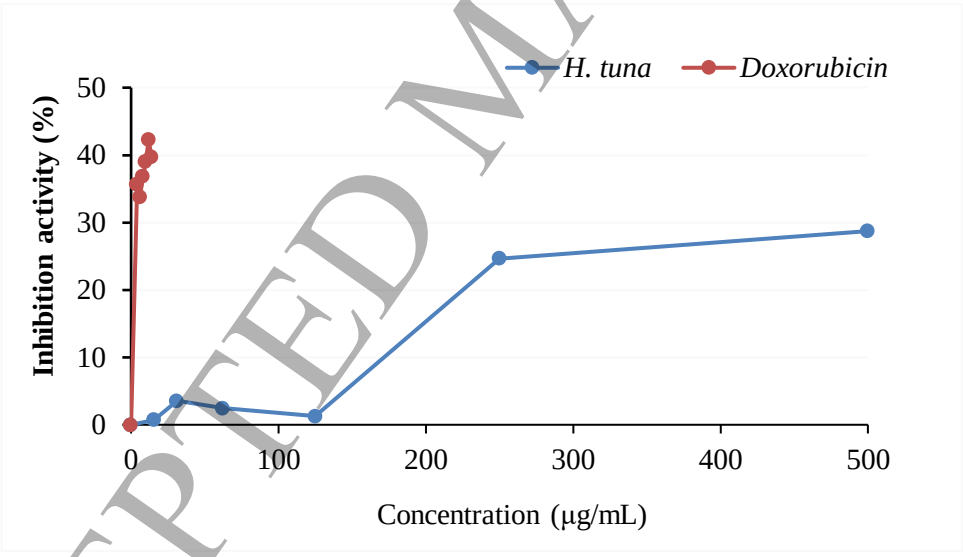


Figure 1. Effect of concentration of *H. tuna* and doxorubicin on inhibition of proliferation of lung cancer cell A549.

Prasetyaningrum *et al.* [28] indicated that the cytotoxicity of a substance based on its IC_{50} is divided into three levels: potential cytotoxic (IC_{50} <100 µg/mL), moderate cytotoxic (100 µg/mL < IC_{50} <1000 µg/mL), and low cytotoxic (IC_{50} >1000 µg/mL). Furthermore, according to the National Cancer Institute [29], a compound can be classified as a strong anticancer agent if its IC_{50} is <20 µg/mL. The cytotoxicity test on the crude extract of *H. tuna* showed low cytotoxic values (IC_{50} value of 2771 µg/mL). A substance with low cytotoxicity can be used

as a chemo-preventive agent. The chemo-preventive ability indicates that the crude extracts of *H. tuna* can be used to prevent and hinder the growth of cancer cells and also trigger apoptosis.

Previous research reported the cytotoxicity of brown seaweed fucoidan extracted from *Turbinaria conoides* species against A549 with IC_{50} of 396.46 μg/mL [30]. Polysaccharide from *Caulerpa taxifolia* showed anticancer acitivity against A549 with a relative IC_{50} of 45.44 μg/mL [31]. Methanol extract of brown algae *Hormophysa cuneiformis* has anticancer activity against A549 with IC_{50} of 40.97 μg/mL [32]. Factors that can affect the content and activity of bioactive metabolites include sampling location or habitat, genetic variation, sampling time, evolution, and environmental conditions [33].

Doxorubicin is an anticancer medicine and an important agent for the therapy of malignant breast cancer [34]. The anticancer action of doxorubicin has been described with various molecular pathways, covering the interaction mechanism of doxorubicin with DNA, DNA-related enzymes, and cell membranes [35]. Another study has shown that *Cladosiphon okamuranus* fucoidan has strong antiproliferative and apoptotic reactions on MCF-7 cells in certain doses and does not affect normal cell proliferation in human mammalian epithelial cells [36]. The cell pattern is a process that requires high energy and involves four sequential stages that change from the stationary stage (G0 stage) to the proliferation stage (G1, S, G2, and M stage) and return to rest [37]. Fucoidan increases the population of hepatocarcinoma (Huh7) cells at the G0/G1 stage and decreases their population at the S stage; this result indicates that fucoidan can induce the cell pattern to persist at the G0/G1 stage [38].

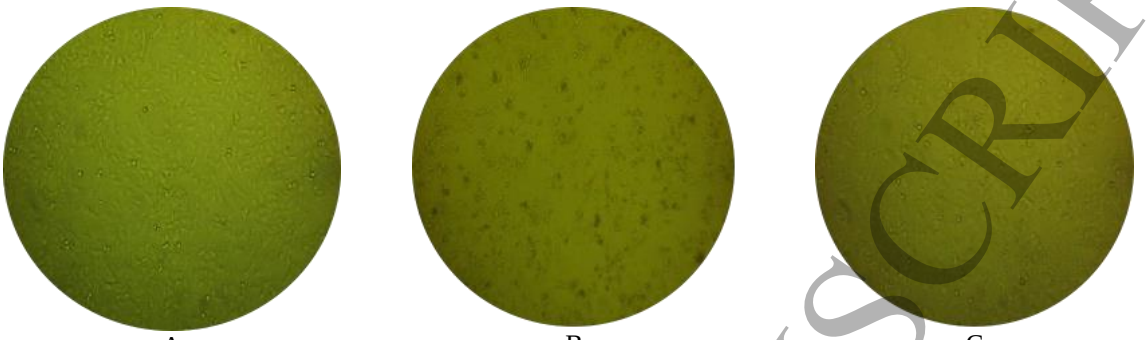
Table 1. IC_{50} values of *H. tuna* extract and doxorubicin against cancer cells A549

Sample	**IC_{50} (μg/mL)**
H. tuna extract	2771[a]
Doxorubicin	24.13[b]

[a/b]Different letters show a significant difference ($p<0.05$)

The differences in the morphological attributes of A549 to *H. tuna* extract and not exposed to *H. tuna* extract are illustrated in Figure 2. The morphological characteristics of A549 exposed to *H. tuna* extract and the control cells not exposed to *H. tuna* extract differed. The morphological attributes of MCF-7 cells in the control cells not exposed to *H. tuna* extract were observed as an irregular polygonal and attached to the substrate. The morphological characteristics of the cells that were exposed to *H. tuna* extract varied, that is, the cells shrank, were round, and had limited distribution patterns compared with those of the control cells. This

change in shape was consistent with that observed by Kim *et al.* [39] who stated that MC3T3 osteoblast cells exposed to fucoidan for 4 h have altered morphological characteristics, i.e., from an irregular shape to a round form with smaller sizes.



A B C

Figure 2. Morphology of A549 lung cancer cells without sample treatment (A), given a sample of *H. tuna* extract 250 µg/mL (B), and given a standard doxorubicin 14 µg/mL (C).

3.3 Phytochemical Content. The phytochemical test aims to identify chemical compounds in samples such as flavonoids, steroids, saponins, tannins, and alkaloids. Many of these chemical compounds are found in seaweed. The results of the phytochemical test were shown in Table 2.

Table 2. Phytochemical analysis of *H. tuna* crude extract

Phytochemicals	Result	Indicator
Flavonoid	++	Yellow/orange color
Steroid	+++	Blue-green color
Triterpenoid	-	Red – purple color
Saponin	-	Foam
Alkaloid	+	Orange precipitate
Tannin	-	Blue-black color

+ : low, ++ : moderate, +++ : high

According to Nome *et al.* [15], flavonoids were found in almost all types of green macroalgae but with different levels, such as *Codium* sp., *Caulerpa* sp., and *Ulva* sp. Similarly, the steroids found in the green macroalgae *Caulerpa* sp., *Halimeda* sp., *Enteromorpha* sp., and *Codium* sp. Alkaloids are also found in green macroalgae such as *Ulva* sp. and *Caulerpa* sp., but little was found in *Halimeda* sp., *Enteromorpha* sp., and *Codium* sp. Gazali *et al.* [40] reported that alkaloids, flavonoids, saponins, and tannins were found in the macroalga *Chaetomorpha crassa*. Based on the research of Widowati *et al.* [22], *Gracilaria salicornia* contains flavonoids, saponins, and steroids, *Halimeda gracilis* contains steroids and saponins,

and *H. macroloba* contains flavonoids and steroids. Gazali *et al*. [12] reported that *H. opuntia* seaweed contains alkaloids, steroids, saponins, flavonoids, phenols, and tannins. Gazali *et al*. [13] reported that the phytochemical test results showed that the *H. tuna* fractions were positive for alkaloids, flavonoids, steroids, and phenol hydroquinone compounds. Flavonoids are secondary metabolites with anticancer activity [41] because these compounds contain quercetin, genistein, or flavopiridol which can be used as cancer drugs [42]. Flavonoids as anticancer have a mechanism of inhibition of DNA topoisomerase I/II activity, decreased expression of Bcl-2 and Bcl-xl genes, and activation of endonucleases [43]. Flavonoids also have the biological ability to chelate metals, inhibiting cancer cell growth [44]. Flavonoids are polar and are mostly produced from green seaweed, so these compounds are generally easily soluble in polar solvents such as methanol [45].

Steroids are non-polar secondary metabolites, so they are easily extracted by polar solvents such as methanol [15]. Steroids have anticancer activity as these compounds have aromatase enzymes and sulfatase inhibitors that can inhibit the growth of cancer cells [46]. Steroids, as anticancer agents, damage mitochondrial membrane permeability in cancer cells and cause cell death or necrosis [47]. In addition, steroids can also capture reactive species such as superoxide and chelate metals [48]. The content of chemical compounds in seaweed can be influenced by environmental factors where it grows because the bioactive compounds formed are a natural response to environmental conditions where they grow, resulting in various types of chemical compounds. The ability of seaweed to produce secondary metabolites that are bioactive compounds can occur due to extreme environmental conditions [15].

3.4 GC-MS Analysis. GC-MS analysis showed a GC spectra chromatogram with seven peaks (Figure 3) representing the bioactive compounds interacting with the GC column. The peak obtained was only a little and not too high, with the results of comparison with the database having a slight similarity. The bioactive activity and utilization of compounds were obtained from the NCBI web and previous studies. Compounds belonging to the flavonoid group were flemichapparin A [49]. The steroids identified in the extract consisted of stigmasta, androst-4-ene-3,17-dione, estra-1,3,5(10)-trien-17-one, 5-alpha-androstan-17-one, and 1-docosanol [50]. Some compounds that include fatty acids include palmitic acid, hexadecanoic acid, octadecanoic acid, lauric acid, 4-hexenoic acid, and dodecanoic acid [46]. The list of information on the identified compounds and the activity of the metabolite compounds from the H. tuna extract is explained further in Table 3.

Table 3. Results of identification of compound components of *H. tuna* methanol extract

Peak	RT	Area (%)	Component	Group	Activity	SI
1	12.308	32.34	stigmasta-5,22-dien-3-ol	Steroid	Antioxidant, antimycobacterial (tuberculosis), Anticancer, inhibition of chemocarcinogen [51]	19
2	13.572	7.73	androst-4-ene-3,17-dione	Steroid	Osteoporosis, antiinfectives, hyperglycemia (antidiabetic) [52]	21
3	18.010	5.03	1-docosanol	Steroid	Antiviral [53]	57
4	19.683	0.01	1-hexadecanol	Fatty Alcohol	Antioxidant, antimicrobial [54]	61
5	20.441	27.41	14-beta-h-pregna	Steroid	Cancer Prevention [55]	63
6	20.883	6.78	dodecanoic acid	Fatty Acid	Antimicrobial, relieve neuro-inflammatory [56]	34
7	21.065	20.73	hexadecanoic acid	Fatty Acid	Anti-inflammatory, antiviral, antioxidant [57]	70

RT: Retention Time, SI: Similarity Index

The activity of volatile compounds, as listed in Table 2, many compounds have anticancer-related bioactivity. The main compound with a large percentage of area is found at peaks 1, 5, and 7, with an area of 32.34%, 27.41%, and 20.73%, respectively. According to Singla and Dubey [58], in predicting compounds using GC-MS, if the similarity value is low (SI<90%) then the component should not be considered because it is less accurate. In this study, the compound with the greatest similarity index was 70, so the compound with the largest percentage area and the greatest similarity was used.

The active compound in peak 1 is stigmasta-5,22-dien-3-ol, with activities including antioxidant, antimycobacterial (anti-tuberculosis bacteria), anti-inflammatory, anticancer, and inhibition of chemocarcinogens. The compound stigmasta-5,22-dien-3-ol belongs to the stigmasteroid group [59]. Stigmasta-5,22-dien-3-ol has been found in the genus *Halimeda* seaweed, precisely in *H. opuntia*, with a percentage of 54.74% as the most dominant compound [60]. In this study, the stigmasta compound only had an SI of 19 so it might not be accurate and have little effect on anticancer activity.

The active compounds in peak 5 include 14-beta-H-pregna with a similarity of 63, which has antidiabetic and cancer-preventive properties. Compound 14-beta-H-pregna belongs to steroids [61]. Compound 14-beta-H-pregna was found with an area of 55% in green seaweed extract *Chlorella vulgaris* [62]. Compound 14-beta-H-pregna is a component of the medicinal plant *Verbascum pseudoholotricum* or mullein with a similarity of 98. Mullein has antioxidant, anti-inflammatory and anti-bacterial activity [63].

Compounds in peak 7 include hexadecanoic acid, octadecanoic acid, dodecanoic acid, and octadecane, a group of fatty acids. Fatty acid bioactivity includes anti-inflammatory, antiviral, antioxidant, antimicrobial, and antibiotic [64]. Fatty acids function as antioxidants so that they can reduce reactive oxygen species and act as preventive agents for diseases caused by reactive oxygen species, such as cancer [37].

Research by Nazaruddin *et al.* [60] on the GC-MS test proved the presence of Hexadecanoic acid in *H. opuntia*. The hexadecanoic acid in Halimeda has antioxidant effects and is cytotoxic against the colorectal cancer cell line HCT-116. The retention time of Hexadecanoic acid in this study was 50.91 min. Nazaruddin *et al.* [11] researched *H. macroloba* using the GC-MS test with the Shimadzu QP2010 Plus GC-MS system. One of the compounds found is hexadecanoic acid. Hexadecanoic acid retention time at two different peaks had values of 21.039 and 20.548 min, respectively. The RT value of the GC-MS test on *H. tuna* in this study for hexadecanoic acid had a retention time of 21.065 min with a similarity index of 70 so it was more similar to the results of the GC-MS test on *H. macroloba.*

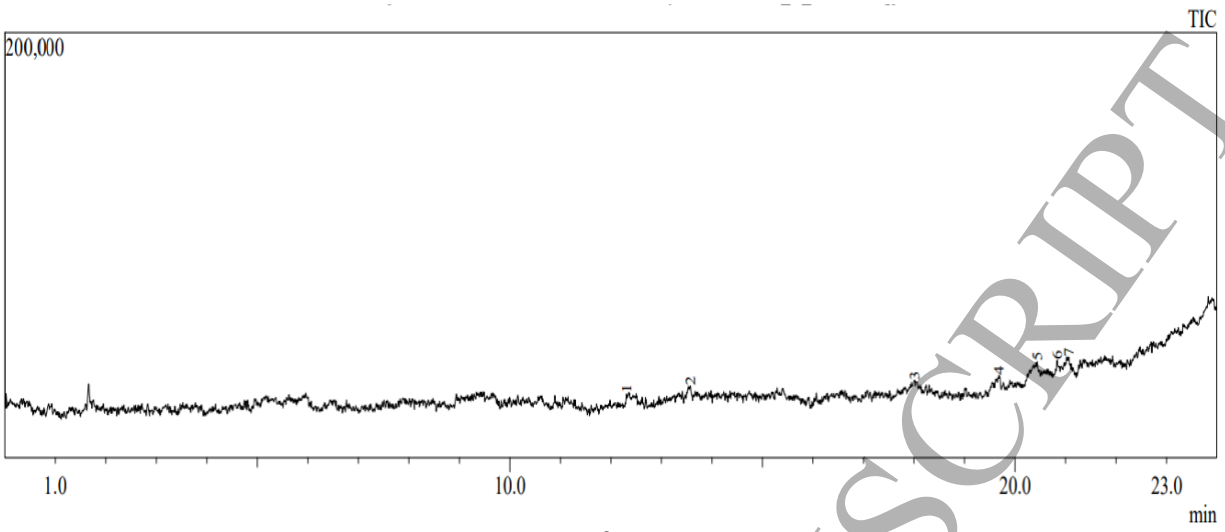


Figure 3. Chromatogram of *H. tuna* methanolic extract

The *H. tuna* extract contains several fatty acid compounds. This is because, in addition to secondary metabolites, seaweed also contains primary metabolites such as protein, carbohydrates, fat, crude fiber, macro minerals, and several vitamins. Differences in the content of chemical compounds in seaweed can be influenced by the type of species and their habitat [15]. Secondary metabolites have been shown to have high bioactivity. However, fatty acids are also known to have antioxidant activity [65], so they are thought to have the potential to have cytotoxic activity. According to Asbanu *et al*. [66], several fatty acids have antioxidant bioactivity such as octadecanoic acid (stearic acid), hexadecanoic acid (palmitic acid), tetradecanoic acid (myristic acid), and 9-octadecenoic acid (oleic acid). In general, the *Halimeda* genus shifts the production of protein and fat primary metabolites to increase the production of halimedatrial and halimedatetraacetate secondary metabolites, so that the bioactive compounds of these secondary metabolites are higher than their primary metabolites [67]. However, this is also influenced by environmental conditions where it grows, resulting in a variety of compound content [68]. In addition, the solvent used is methanol, which is a universal polar solvent so that it can attract all compounds, both polar and non-polar compounds, such as fats [69]. Methanol is also one of the most widely used solvents in the extraction process of organic compounds such as oils or fats [70], so fatty acids can be carried away in the extraction process.

4. CONCLUSIONS

H. tuna methanolic extract was obtained in 0.17±0.04% yield. *H. tuna* extract had cytotoxic activity against lung cancer cells (A549) with IC_{50} 2771 µg/mL and potentially can be used as a chemo-preventive agent. Based on cytomorphological observations, changes in the morphology of cancer cells were seen before and after being treated with *H. tuna* extract samples. Metanolic extract of *H. tuna* have contents palmitic acid, oleic acid, palmitoleic acid, myristic acid, and stearic acid.

REFERENCES

[1] J. Joseph and L. W. A. Rotty. (2020). "Kanker paru: laporan kasus". *Medical Scope Journal.* **2** (1): 17–25. 10.35790/msj.v2i1.31108.

[2] R. L. Siegel, K. D. Miller, and A. Jemal. (2016). "Cancer statistics, 2016". *CA: A Cancer Journal for Clinicians.* **66** (1): 7-30. 10.3322/caac.21332.

[3] L. Warrington, K. Absolom, M. Conner, I. Kellar, B. Clayton, M. Ayres, and G. Velikova. (2019). "Electronic Systems for Patients to Report and Manage Side Effects of Cancer Treatment: Systematic Review". *Journal of Medical Internet Research.* **21** (1): e10875. 10.2196/10875.

[4] S. N. Syed Mohammad Salleh, M. Farooqui, S. Gnanasan, and M. Karuppannan. (2021). "Use of complementary and alternative medicines (CAM) among Malaysian cancer patients for the management of chemotherapy related side effects (CRSE)". *Journal of Complementary and Integrative Medicine.* **18** (4): 805-812. 10.1515/jcim-2020-0205.

[5] T. H. Ranahewa, A. D. Premarathna, R. M. K. K. Wijesundara, V. Wijewardana, A. P. Jayasooriya, and R. P. V. J. Rajapakse. (2020). "Biochemical Composition and Anticancer Effect of Different Seaweed Species (In-vitro and In-vivo Studies)". *Sustainable Marine Structures.* **1** (2): 5-11. 10.36956/sms.v1i2.94.

[6] J. Debbarma, B. Madhusudana Rao, L. N. Murthy, S. Mathew, G. Venkateshwarlu, and C. N. Ravishankar. (2016). "Nutritional profiling of the edible seaweeds Gracilaria edulis, Ulva lactuca and Sargassum sp". *Indian Journal of Fisheries.* **63** (3): 81–7. 10.21077/ijf.2016.63.3.60073-11.

[7] J. Praiboon, S. Palakas, T. Noiraksa, and K. Miyashita. (2017). "Seasonal variation in nutritional composition and anti-proliferative activity of brown seaweed, Sargassum oligocystum". *Journal of Applied Phycology*. **30** (1): 101-111. 10.1007/s10811-017-1248-6.

[8] A. R. Ganesan, U. Tiwari, and G. Rajauria. (2019). "Seaweed nutraceuticals and their therapeutic role in disease prevention". *Food Science and Human Wellness*. **8** (3): 252-263. 10.1016/j.fshw.2019.08.001.

[9] W. Widowaty. (2018). "Aktivitas antibakteri ekstrak kasar Sargassum sp. dari Pantai Sayang Heulang, Garut Jawa Barat". *Agroscience.* **8** (2): 268–274.

[10] Y. Yoshie, W. Wand, Y. P. Hsieh, and T. Suzuki. (2002). "Compositional difference of phenolic compounds between two seaweeds, Halimeda spp". *Journal of Tokyo University of Fisheries.* **88** : 21–24.

[11] M. F. Nazarudin, A. Isha, S. N. Mastuki, N. M. Ain, N. F. Mohd Ikhsan, A. Z. Abidin, and M. Aliyu-Paiko. (2020). "Chemical Composition and Evaluation of the alpha-Glucosidase Inhibitory and Cytotoxic Properties of Marine Algae Ulva intestinalis, Halimeda macroloba, and Sargassum ilicifolium". *Evidence-Based Complementary and Alternative Medicine.* **2020** : 2753945. 10.1155/2020/2753945.

[12] M. Gazali, Nurjanah, and N. P. Zamani. (2019). "The screening of bioactive compound of the green algae Halimeda macroloba (Decaisne, 1841) as an antioxidant agent from Banyak Island Aceh Singkil". *IOP Conference Series: Earth and Environmental Science.* **348** (1): 012043. 10.1088/1755-1315/348/1/012043.

[13] M. Gazali, Nurjanah, and N. P. Zamani. (2019). "The Screening of Green Algae Halimeda opuntia (Linnaeus) as an Antioxidant from the Coast of West Aceh". *Jurnal Ilmu Pertanian Indonesia.* **24** (3): 267-272. 10.18343/jipi.24.3.267.

[14] M. Gazali, O. Jolanda, A. Husni, Nurjanah, F. A. A. Majid, Zuriat, and R. Syafitri. (2023). "In Vitro alpha-Amylase and alpha-Glucosidase Inhibitory Activity of Green Seaweed Halimeda tuna Extract from the Coast of Lhok Bubon, Aceh". *Plants (Basel).* **12** (2): 10.3390/plants12020393.

[15] W. Nome, Y. Salosso, and C. B. Eoh. (2019). "Analisis metabolit sekunder dan kandungan nutrisi dari makroalga hijau (Chlorophyceae) di perairan Teluk Kupang". *Jurnal Aquantik.* **2** (1): 100–112.

[16] P. Melati. (2021). "Uji aktivitas antioksidan, sitotoksisitas dan gc-ms ekstrak metanol alga hijau Boergesenia forbesii (harvey) feldmann dari pantai panjang bengkulu".

Jurnal Pengelolaan Laboratorium Sains dan Teknologi. **1** (1): 10-24. 10.33369/labsaintek.v1i1.15432.

[17] R. M. Puc, D. Robledo, and Y. F. Pelegrin. (2009). "In vitro cytotoxic and antiproliferative activities of marine macroalgae from Yucatan, Mexico". *Ciencias Marinas.* **35** (4): 345–358. 10.7773/cm.v35i4.1617.

[18] G. Sanger, B. E. Kaseger, L. K. Rarung, and L. Damongilala. (2018). "Potensi beberapa Jenis Rumput Laut sebagai Bahan Pangan Fungsional, Sumber Pigmen dan Antioksidan Alami". *Jurnal Pengolahan Hasil Perikanan Indonesia.* **21** (2): 208–217. 10.17844/jphpi.v21i2.22841.

[19] W. Safia, Budiyanti, and Musrif. (2020). "Kandungan Nutrisi dan Bioaktif Rumput Laut (Euchema cottonii) dengan Metode Rakit Gantung pada Kedalaman Berbeda". *Jurnal Pengolahan Hasil Perikanan Indonesia.* **23** (2): 261-271. 10.17844/jphpi.v23i2.29460.

[20] A. Husni, B. Pamungkas, E. Sinurat, and A. Isnansetyo. (2021). "Characteristics and Cytotoxic Activity of Fucoidan from the Brown Seaweed Sargassum hystrix against MCF-7 Breast Cancer Cells". *Tropical Journal of Natural Product Research.* **5** (3): 564-569. 10.26538/tjnpr/v5i3.24.

[21] D. O. Lubis, M. Hendri, and Rozirwan. (2020). "The potential of bioactive compounds of Halimeda micronesica and Halimeda macroloba species of seaweeds, obtain from Maspari Island, South Sumatra to express antioxidant activities, and the phytochemical screening of their active extracts". *International Journal of Marine Science.* **10** (6): 1–7.

[22] R. Widowati, S. Handayani, and R. Suprihatin. (2021). "Phytochemicals and antioxidant of metanol extract of Gracilaria salcornia, Halimeda gracilis, Halimeda macroloba, and Hypnea asperi from Tidung Island Coastal Region". *European Journal of Molecular and Clinical Medicine.* **08** (1): 896–907.

[23] E. N. Hidayah. (2017). "Analisis metabolomik padi hitam (Oryza sativa L)". *Thesis.*

[24] A. Husni, P. Tiara, S. Ustadi, A.g, and A. E.n. (2018). "In vitro antidiabetic activity of Sargassum hystrix and Eucheuma denticulatum fromYogyakarta Beach of Indonesia". *Proceedings of the Pakistan Academy of Sciences: B. Lifeand Environmental Sciences.* **55** (3): 1–8.

[25] A. F. Muzaki, W. A. Setyati, S. Subagiyo, and R. Pramesti. (2018). "Aktivitas antioksidan ekstrak rumput Laut Halimeda macroloba dari pantai teluk awur, jepara, jawa tengah". *Jurnal Enggano.* **3** (2): 144-155. 10.31186/jenggano.3.2.144-155.

[26] S. Purwaningsih and E. Deskawati. (2021). "Karakteristik dan Aktivitas Antioksidan Rumput Laut Gracilaria sp. Asal Banten". *Jurnal Pengolahan Hasil Perikanan Indonesia*. **23** (3): 503-512. 10.17844/jphpi.v23i3.32808.

[27] L. H. Nurani, S. Widyarini, and A. Mursyidi. (2015). "Uji Sitotoksik Dan Uji Kombinasi Fraksi Etil Asetat Ekstrak Etanol Akar Pasak Bumi (Eurycoma Longifolia Jack.,) Dan Doksorubisin Pada Sel Limfosit". *Journal of Tropical Pharmacy and Chemistry*. **3** (2): 138-147. 10.25026/jtpc.v3i2.100.

[28] P. Wiji Prasetyaningrum, A. Bahtiar, and H. Hayun. (2018). "Synthesis and Cytotoxicity Evaluation of Novel Asymmetrical Mono-Carbonyl Analogs of Curcumin (AMACs) against Vero, HeLa, and MCF7 Cell Lines". *Scientia Pharmaceutica*. **86** (2): 1–13. 10.3390/scipharm86020025.

[29] N. C. Institute. (2017). "Breast cancer treatment (PDQ)-health professional version".

[30] R. C. Santhanam, S. A. M. Yacoob, and A. Venkatraman. (2022). "In vitro cytotoxicity assay of fucoidan extracted from Turbinaria conoides against cancer cell lines MCF7, A549, and normal cell line L929". *Brazilian Journal of Pharmaceutical Sciences*. **28** (19542) : 1–6.

[31] A. M. Bayro, J. K. Manlusoc, R. Alonte, C. Caniel, P. Conde, and C. Embralino. (2021). "Preliminary characterization, antioxidant and antiproliferative properties of polysaccharide from Caulerpa taxifolia". *Pharmaceutical Sciences and Research*. **8** (1): 30 – 36.

[32] N. A. H. K. Osman, A. A. Siam, I. M. El-Manawy, and Y. J. Jeon. (2020). "Anticancer Activity of a scarcely investigated Red Sea Brown Alga Hormophysacuneiformis against HL60, A549, HCT116 and B16 Cell Lines". *Egyptian Journal of Aquatic Biology & Fisheries*. **24** (1): 497 – 508.

[33] A. Mayasri. (2021). "Potensi beberapa jenis rumput laut di Aceh (studi kasus skrining fitokimia dan aktivitas antioksidan". *Lantanida Journal*. **9** (1): 83–92.

[34] K. Greish, M. Fateel, S. Abdelghany, N. Rachel, H. Alimoradi, M. Bakhiet, and A. Alsaie. (2018). "Sildenafil citrate improves the delivery and anticancer activity of doxorubicin formulations in a mouse model of breast cancer". *Journal of Drug Targeting* **26** (7): 610-615. 10.1080/1061186X.2017.1405427.

[35] I. Borisev, J. Mrdanovic, D. Petrovic, M. Seke, D. Jovic, B. Srdenovic, N. Latinovic, and A. Djordjevic. (2018). "Nanoformulations of doxorubicin: how far have we come and where do we go from here?". *Nanotechnology*. **29** (33): 332002. 10.1088/1361-6528/aac7dd.

[36] Y. Lin, X. Qi, H. Liu, K. Xue, S. Xu, and Z. Tian. (2020). "The anti-cancer effects of fucoidan: a review of both in vivo and in vitro investigations". *Cancer Cell International.* **20** (154): 154. 10.1186/s12935-020-01233-8.

[37] C. Norbury and P. Nurse. (1992). "Animal cell cycles and their control". *Annual Review of Biochemistry.* **61** : 441-70. 10.1146/annurev.bi.61.070192.002301.

[38] T. Nagamine, K. Hayakawa, T. Kusakabe, H. Takada, K. Nakazato, E. Hisanaga, and M. Iha. (2009). "Inhibitory effect of fucoidan on Huh7 hepatoma cells through downregulation of CXCL12". *Nutrition and Cancer.* **61** (3): 340-7. 10.1080/01635580802567133.

[39] E. J. Kim, S. Y. Bu, M. K. Sung, and M. K. Choi. (2013). "Effects of silicon on osteoblast activity and bone mineralization of MC3T3-E1 cells". *Biological Trace Element Research.* **152** (1): 105-12. 10.1007/s12011-012-9593-4.

[40] M. Gazali, N. P. Zamani, and Nurjanah. (2019). "The potency of green algae Chaetomorpha crassa Agardh as antioxidant agent from the coastal of Lhok Bubon, West Aceh". *IOP Conference Series: Earth and Environmental Science.* **278** (1): 012029. 10.1088/1755-1315/278/1/012029.

[41] M. Syahril, O. Roshani, N. Hasyimah, M. Hfiz, M. D. Sharida, and H. Y. Ahmed. (2011). "Screening of anticancer activities of crude extracts of unicellular green algae (Spirulina) on selected cancer cell lines". *International Conference on Applied Science, Mathematics, and Humanities*.

[42] D. Ravishankar, A. K. Rajora, F. Greco, and H. M. Osborn. (2013). "Flavonoids as prospective compounds for anti-cancer therapy". *The International Journal of Biochemistry & Cell Biology*. **45** (12): 2821-31. 10.1016/j.biocel.2013.10.004.

[43] W. Ren, Z. Qiao, H. Wang, L. Zhu, and L. Zhang. (2003). "Flavonoids: promising anticancer agents". *Medicinal Research Reviews.* **23** (4): 519-34. 10.1002/med.10033.

[44] T. Y. Wang, Q. Li, and K. S. Bi. (2018). "Bioactive flavonoids in medicinal plants: Structure, activity and biological fate". *Asian J Pharm Sci.* **13** (1): 12-23. 10.1016/j.ajps.2017.08.004.

[45] F. J. Rachmawaty, D. A. C. Mahardina, B. Nirwani, T. Nurmasitoh, and E. T. Bowo. (2010). "Manfaat sirih merah (Piper crocatum) sebagai agen antibacterial terhadap bakteri gram positif dan gram negatif". *Jurnal Kedokteran dan Kesehatan Indonesia.* **1** (7): 10–25.

[46] P. S. Sirait, I. Setyaningsih, and K. Tarman. (2019). "Anticancer Activity of Spirulina Cultivated in Walne and Organic Media". *Jurnal Pengolahan Hasil Perikanan Indonesia*. **22** (1): 50–59. 10.17844/jphpi.v22i1.25876.

[47] Z. A. Zakaria, A. M. Mohamed, N. S. Jamil, V. Rofiee, M. N. Smochit, A. Zuraini, A. K. Arifah, and M. R. Sulaiman. (2011). "In-vitro cytotoxic and antioxidant properties of the aqueous, chloroform and metanol extracts of Dicranopteris linearis leaves". *African Journal of Biotechnology*. **10** (2): 273–282. 10.5897/AJB10.423.

[48] G. Topcu, A. Ertas, U. Kolak, M. Ozturk, and V. Ulubelen. (2007). "Antioxidant activity tests on novel triterpenoids from Salvia macrochlamys". *Archive for Organik Chemistry*. **7** : 195–208.

[49] C. M. M. Santos and A. M. S. Silva. (2020). "The Antioxidant Activity of Prenylflavonoids". *Molecules*. **25** (3). 10.3390/molecules25030696.

[50] A. V. Baranovsky and R. P. Litvinovskaya. (2019). "1H and 13C NMR Spectral Characteristics of 15-Substituted Pregn-5-Ene and Androst-5-Ene Steroid Compounds". *Journal of Applied Spectroscopy*. **86** : 867–876.

[51] M. Zahid, M. Arif, M. A. Rahman, K. Singh, and M. Mujahid. (2018). "Solvent Extraction and Gas Chromatography-Mass Spectrometry Analysis of Annona squamosa L. Seeds for Determination of Bioactives, Fatty Acid/Fatty Oil Composition, and Antioxidant Activity". *Journal of Dietary Supplements*. **15** (5): 613-623. 10.1080/19390211.2017.1366388.

[52] K. Yildirim, A. Kuru, and Ş. Yılmaz. (2018). "Biotransformation of Testosterone by Ulocladium Chartarum Mrc 72584". *Journal of Chemical Research*. **42** (8): 444-446. 10.3184/174751918x15341764332783.

[53] D. T. Leung and S. L. Sacks. (2004). "Docosanol: a topical antiviral for herpes labialis". *Expert Opin Pharmacother*. **5** (12): 2567-71. 10.1517/14656566.5.12.2567.

[54] B. Pejin, D. Nakarada, M. Novakovic, V. Tesevic, A. Savic, K. Radotic, and M. Mojovic. (2014). "Antioxidant volatiles of the freshwater bryozoan Hyalinella punctata". *Natural Product Research*. **28** (18): 1471-5. 10.1080/14786419.2014.905565.

[55] Handayani, D. D. Wulandari, and D. D. Wulansari. (2022). "Phytochemical screening, antioxidant activity and cytotoxicity assay from noni juice and fermented noni (Morinda citrifolia L.)". *Bali Medical Journal*. **11** (3): 1168-1171. 10.15562/bmj.v11i3.3534.

[56] D. Sarova, A. Kapoor, R. Narang, V. Judge, and B. Narasimhan. (2010). "Dodecanoic acid derivatives: Synthesis, antimicrobial evaluation and development of one-target and multi-target QSAR models". *Medicinal Chemistry Research.* **20** (6): 769-781. 10.1007/s00044-010-9383-5.

[57] T. Ganesan, M. Subban, D. B. Christopher Leslee, S. B. Kuppannan, and P. Seedevi. (2022). "Structural characterization of n-hexadecanoic acid from the leaves of Ipomoea eriocarpa and its antioxidant and antibacterial activities". *Biomass Conversion and Biorefinery.* 10.1007/s13399-022-03576-w.

[58] R. K. Singla and A. K. Dubey. (2019). "Phytochemical profiling, GC-MS analysis and α-amylase inhibitory potential of ethanolic extract of Cocos nucifera Linn. Endocarp. Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune". *Endocrine & Metabolic Disorders.* **19** (4): 419–442.

[59] M. Loori, I. Sourinejad, and M. Nazemi. (2021). "Identification and investigation of antibacterial effects of steroidal fraction from the marine sponge Axinella sinoxea Alvarez & Hooper, 2009 in Larak island, the Persian Gulf". *Fisheries Science and Technology.* **10** (2): 164–172.

[60] M. F. Nazarudin, I. S. M. Yasin, N. Mazli, A. R. Saadi, M. H. S. Azizee, M. A. Nooraini, N. Saad, U. T. Ferdous, and I. M. Fakhrulddin. (2022). "Preliminary screening of antioxidant and cytotoxic potential of green seaweed, Halimeda opuntia (Linnaeus) Lamouroux". *Saudi Journal of Biological Sciences.* **29** (4): 2698-2705. 10.1016/j.sjbs.2021.12.066.

[61] D. L. Zhang, Y. H. Feng, Z. Y. Liang, Q. Lin, and J. Xu. (2012). "Chemical composition of essential oil from fruit of Ficus altissima". *Advanced Materials Research.* **554** : 1125–1128. 10.4028/www.scientific.net/AMR.554-556.1125.

[62] H. P. Kusumaningrum, M. Zainuri, H. Endrawati, B. D. Loka, I. N. Widiasa, and E. Sulistyowati. (2018). "Chemical compounds in essential oil of nutmeg leaves (Myristica fragrans) from Batang Indonesia". *Journal of Physics: Conference Series.* 012096.

[63] E. Yabalak, F. Ibrahim, E. A. E. Eliuz, A. Everest, and A. M. Gizir. (2020). "Evaluation of chemical composition, trace element content, antioxidant and antimicrobial activities of Verbascum pseudoholotrichum". *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology.* **156** (2): 313-322. 10.1080/11263504.2020.1852332.

[64] A. Ruiz-Rodriguez, G. Reglero, and E. Ibanez. (2010). "Recent trends in the advanced analysis of bioactive fatty acids". *Journal of Pharmaceutical and Biomedical Analysis.* **51** (2): 305-26. 10.1016/j.jpba.2009.05.012.

[65] A. V. Novoa, E. R. S. Andrade-Wartha, A. F. Linares, A. M. d. O. e. Silva, M. I. Genovese, A. E. B. González, P. Vuorela, A. Costa, and J. Mancini-Filho. (2011). "Antioxidant activity and possible bioactive components in hydrophilic and lipophilic fractions from the seaweed Halimeda incrassata". *Revista Brasileira de Farmacognosia.* **21** (1): 53-57. 10.1590/s0102-695x2011005000010.

[66] Y. W. A. Asbanu, N. Wijayanti, and E. Kusumo. (2019). "Identifikasi senyawa kimia ekstrak daun sirsak (Annona muricata L.) dan uji aktivitas antioksidannya dengan metode DPPH (2,2-difenil-1-pikrilhidrasil". *Indonesian Journal of Chemical Science.* **8** (3): 153–160. 10.15294/IJCS.V9I2.33525.

[67] G. O. Longo and M. E. Hay. (2014). "Does seaweed–coral competition make seaweeds more palatable?". *Coral Reefs.* **34** (1): 87-96. 10.1007/s00338-014-1230-6.

[68] A. Nurhayati, N. S.n.k, R, and Murdinah. (2017). "Komposisi nutrisi rumput laut Calcareous Halimeda opuntia pada lingkungan perairan Indonesia". *Jurnal Pascapanen dan Bioteknologi Kelautan dan Perikanan.* **12** (1): 13–22.

[69] N. Salamah and E. Widyasari. (2015). "Aktivitas antioksidan ekstrak metanol daun kelengkeng (Euphoria longan (L) Steud.) dengan metode penangkapan radikal 2,2'-difenil-1-pikrilhidrazil". *Pharmaciana.* **5** (1): 25–34. 10.12928/pharmaciana.v5i1.2283.

[70] Y. Gu and F. Jerome. (2013). "Bio-based solvents: an emerging generation of fluids for the design of eco-efficient processes in catalysis and organic chemistry". *Chemical Society Reviews.* **42** (24): 9550-70. 10.1039/c3cs60241a.