The effect of extraction solvent polarity on cytotoxic properties of *Sargassum crassifolium* against B16-F10 melanoma cancer cell model

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Abstract. The prevalence of skin cancer continues to increase from year to year. Skin cancer mainly caused by melanin accumulation on the skin surface (hyperpigmentation) or excessive melanogenesis occurs. The brown macroalgae *S. crassifolium* is one of marine natural resources which is abundant in Lombok, Indonesia coastal areas. However, information regarding its potential bioactive activities remains limited. This study aims to evaluate the effective extraction solvent for *S. crassifolium* for gaining optimum bioactive compounds with promising cytotoxic activity against melanoma cell line. The three variations of the solvent used are ethanol, n-hexane, and ethyl acetate. Some major compounds that were detected by GC-MS analysis in *S. crassifolium* were stated as follow: n-Hexadecanoic acid (Palmitic acid), Hexadecanoic acid, methyl ester (Methyl palmitate), Oleic Acid, Dodecanoic acid, methyl ester, 9-Octadecenoic acid, methyl ester, Tetradecanoic acid, methyl ester. SEA showed the higher amount of methyl palmitate compared to *S. crassifolium* extracted with other solvent. Accordingly, to the major chemical constituent, SEA showed highest IC₅₀ against melanoma cell line (61.26 ± 2.13 μg/mL) compared to SET and SNX. SEA also induced apoptosis which characterized by increase in apoptotic nuclei.

1. Introduction

Premature aging starts from abnormal skin alteration and changes in skin structure such as firmness, smoothness, and decreased ability of skin function and uneven skin color [1]. Alteration in skin color due to the increasing production of melanin content. The formation of melanin induced bytyrosinase enzyme will make the skin color get darker. Excessive production of melanin pigment can cause melanin accumulation on the skin surface (hyper pigmentation) or excessive melanogenesis occurs[2]

For instance, the increased production of melanin can lead to the skin cancer. Several previous study reported that marine macroalgae have potential medicinal uses against cancer. *S. crassifolium* is one of brown macroalgae that was known as an abundant source of diverse bioactive compound to reduce the progressivity of cancer including skin cancer [3]. Nonetheless, the study of this diverse bioactive compound has been very limited due to the lack of knowledge...
related to the optimum processing condition of marine macroalgae. The knowledge of extracting and processing techniques to obtained optimum extraction condition being necessary. More effective extraction technology including the development of optimum extraction solvent may affect higher amount of potential bioactive compound. Extraction solvent optimization greatly affects the extraction yield and secondary metabolites content [4]. The level of polarity of the solvent will affect the extraction process, yield and chromatogram pattern of the extract [5]. Based on these promising impacts, this study aims to evaluate the optimum extraction solvent for *S. crassifolium* on multiple variables including secondary metabolites content and cytotoxic effect of *S. crassifolium* against B16-F10 cell line as skin cancer cell model. Three different solvent which are evaluated in this recent study are ethanol, n-hexane, and ethyl acetate. Ethanol is a polar organic solvent, ethyl acetate and n-hexane are semi-polar and non-polar solvent respectively. Polar solvents will easily attract the content of polar metabolites, as well as non-polar or semi-polar solvents will be able to attract non-polar or semi-polar metabolites [6-7].

2. Material and Methods

2.1 Sample collection and preparation

Macroalgae *Sargassum crassifolium* was collected at Batu Layar coastal area, West Lombok, West Nusa Tenggara (WNT), Indonesia (8°31'04.2”S 116°03'41.9”E). Sampling time is carried out during the time of the lowest tide at intertidal area of Batu Layar in April 2021. After collection, *S. crassifolium* was rinsed with fresh water and further drying at a stable room temperature in range 20 °C – 25 °C. Dried macroalgae then cuts into small pieces to optimize the maceration process. Maceration was done under the term of conditions as follow: a solid–solvent ratio of 1:10 using different solvent polarity (ethanol 96%, n-hexane and ethyl acetate) for 24 hours and replicate in 3 times. The macerated supernatant was separated from the macroalgae powder then enter the evaporation process which aims to remove the solvent using rotary evaporator (Rotavapor R-215, Switzerland) with maximum temperature 50 °C [8-9].

2.2 GC-MS Profiling

The carrier gas used is helium with the flow rate set as follows: injector temperature 320 °C, oven initial temperature 70 °C. The temperature rise rate is 10 °C/min, and the final oven temperature is 310 °C [10-11].

2.3 Cell culture

The B16-F10 cancer cell model (ECACC 92101204) are grown under the following conditions: grown in Dulbecco's modified eagle medium (DMEM) with 10% Fetal Bovine Serum, 2mM Glutamine. Cells were culture and passage after they reach 80-90% confluent. Split sub-confluent cultures (70-80%) 1:2 to 1:4 i.e. seeding at 2-5×10,000 cells/cm² using 0.05% trypsin or trypsin/EDTA; 5% CO2; 37 °C. Cells were seeded to 96 well plate and 35 mm cell culture dish for MTT assays and viability assays respectively [1].

2.4 Cell viability assays

Cell viability assays of melanocyte cell line (*Murine B16-F10*) were determined using the Methyl Thiazolil Tetrazolium (MTT) assay in 96 well plates after *S. crassifolium* extract treatment for 48 hours. Cell counting was performed using Multiskan GO Microplate Reader Thermo Fisher Scientific at 450 nm. The viability of the cells was determined using an equation as follow [12]:

\[
\text{Cell viability (%) } = \frac{((\text{Abs}_{450} \text{ of sample} - \text{Abs}_{450} \text{ of blank}) / (\text{Abs}_{450} \text{ of untreated well} - \text{Abs}_{450} \text{ of blank})) \times 100\%)}
\]

The concentration used in this study is the logarithmic concentration of 10μg/mL, 30μg/mL, 100 μg/mL, 300μg/mL and 1000μg/mL.
2.5 Hoescht33342 staining
Stock solution of Hoescht 33342 was prepared by dissolving 0.1 mg of Hoescht 33342 with 1 mL of PBS and working solution (10 μL stock solution in 2 mL of DMEM) was also prepared. B16-F10 cell lines were cultured in the working solution for 15 minutes at 37°C in 5% CO₂ in air at high humidity. The fluorescence evaluation was performed by exposing the cell culture dish to the UV light and the number of cell that give fluorescence was the primary parameter under an inverted microscope (Zeiss, Gottingen, Germany) [13].

2.6 Statistical analysis
The data obtained will be processed by analysis of variance (ANOVA) with 95% confidence level. If the results show a significant difference, further tests are carried out using Tukey-HSD test, *p* < 0.05 was considered as statistical significant (n = 3 in each group). The data also analyzed with GraphPad Prism 9.2.0 version (GraphPad Software, Inc.) and ImageJ software [12].

3. Results and Discussion
S. crassifolium extensively utilize for it nutrient content and alginate content. Besides, the excessive use of S. crassifolium and other species of brown macroalgae as biofertilizer also reduces polluting our environment. Marine macroalgae especially brown macroalgae possess unique biological activities including cytotoxicity potential. *S. crassifolium* as presented in Figure 1 is one of the brown macroalgae that was known as an abundant source of diverse bioactive compound and chemical constituents. Assessment of the chemical constituent of *S. crassifolium* extracted with three different solvents was determined with GC-MS analysis. Based on the mass spectra in Figure 2, it indicates that there is a separation of peaks at almost the same wavenumber between *S. crassifolium* extracted by n-hexane (SNX) and *S. crassifolium* extracted in ethyl acetate (SEA) but slightly different at *S. crassifolium* extracted by ethanol (SET). Nevertheless, the peak profile of GC-MS chromatogram cannot be used to ensure the presence of the same bioactive constituent between the extracts. Therefore, it is still necessary to provide several other parameters in this GC-MS analysis. Some major compounds that were detected by GC-MS in *S. crassifolium* were state as follow: n-Hexadecanoic acid (Palmid acid), Hexadecanoic acid, methyl ester (Methyl palmitate), Oleic Acid, Dodecanoic acid, methyl ester, 9-Octadecenoic acid, methyl ester, Tetradecanoic acid, methyl ester. The chromatogram in Figure 2 shows that the content of the *S. crassifolium* extract was dominated by compounds of the fatty acid group.

![Figure 1. Sargassum crassifolium. The brown macroalgae including *S. crassifolium* is widely distributed on the intertidal line of the west coastal area of Lombok island, West Nusa Tenggara Indonesia.](image)
Figure 2. GC-MS Chromatogram. Investigation of the chemical constituents in *S. crassifolium* was determined with GC-MS analysis. Some main compounds that were detectable in *S. crassifolium* were: Hexadecanoic acid (Palmitic acid), Hexadecanoic acid, methyl ester (Methyl palmitate), Oleic Acid, Dodecanoic acid, methyl ester, 9-Octadecenoic acid, methyl ester, Tetradecanoic acid, methyl ester.

From the Table 1. below, it revealed that SET, SNX and SEA have one presented primary compound that has been found in highest percentage, n-Hexadecanoic acid (Palmitic acid) and Hexadecanoic acid, methyl ester (Methyl palmitate). Methyl palmitate is the product of esterified form of palmitic acid with higher hidrobocity. However, both are classified into the same fatty acid group with practically the same biological activity [14-15].

The quantity of methyl palmitate (MP) detected in GC-MS are affected by different solvent polarity. Solvent polarity had a significant effect on the amount of methyl palmitate detected GC-MS. It is found to be more efficient to extracted the *S. crassifolium* using semi polar solvent such as ethyl acetate (EA). *S. crassifolium* extracted with EA (SEA) showed the higher amount of methyl palmitate compared to *S. crassifolium* extracted with other solvent. Methyl palmitate is a relatively hidrofobic fatty acid [16]. However, methyl palmitate has higher polarity than n-hexane. This is one of the probable reason for better dissolution of methyl palmitate in semi-polar solvent such as ethyl acetate [17-18]. Moreover, several other studies have also shown that ethyl acetat also results in higher chemical substituent present in extract.
Table 1. Chemical constituent present in GLE analysed by GCMS.

| SET          | SNX          | SEA          |
|--------------|--------------|--------------|
| n-Hexadecanoic acid | 24.44        | Hexadecanoic acid, methyl ester | 32.89 |
| Oleic Acid   | 22.74        | Dodecanoic acid, methyl ester | 21.58 |
| 9-Octadecenoic acid, methyl ester, (E)- | 16.01 | Tetradecanoic acid, methyl ester | 15.29 |
| Hexadecanoic acid, methyl ester | 9.12 | Octadecanoic acid, methyl ester | 8.05 |
| 9-Octadecenoic acid (Z)-, methyl ester | 6.70 | Cycloheptasiloxane, tetradecamethyl- | 4.91 |
| 9-Octadecenoic acid, methyl ester, (E)- | 3.23 | Decanoic acid, methyl ester | 2.70 |
| 9,12-Octadecadienoic acid, methyl ester | 3.04 | Cyclohexasiloxane, dodecamethyl- | 2.32 |
| Octadecanoic acid, methyl ester | 2.66 | HEXADECAMETHYL CYCLOOCTASILOXANE | 2.11 |
| Dodecanoic acid, methyl ester | 2.37 | S-Methyl methanethiosulphonate | 1.76 |
| Stigmast-5-en-3-ol, olate | 2.00 | 9-Octadecenoic acid (Z) -, methyl ester | 1.75 |
| Tetradecanoic acid, methyl ester | 1.87 | Methane, (methylsulfanyl)(methylthio) | 1.65 |
| Tetradecanamide | 0.98 | Cyclopentasiloxane, decamethyl- | 1.48 |
| Hexadecanoic acid, methyl ester | 0.82 | CYCLONONASILOXANE,OCTADECAME- | 1.41 |
| Benzenemethanamine, N- (phenylmethylene)- | 0.74 | Phenol, 2,6-bis(1,1-dimethylethyl)-4-methyl- | 1.14 |
| TETRACOSAMETHYLCYCLODODECASILXANE | 0.61 | 9-Octadecenoic acid (Z)-, methyl ester | 0.94 |
| Tetracosanoic acid, methyl ester | 0.57 | Methane, (methylsulfanyl)(methylthio)- | 0.88 |
| Tricosanoic acid, methyl ester | 0.56 |                  |     |
| Cholest-5-en-3-ol (3.beta.) , carbonochloridate | 0.54 |                  |     |
| Cyclononasiloxane, octadecamethyl- | 0.52 |                  |     |
| TETRACOSAMETHYLCYCLODODECASILXANE | 0.47 |                  |     |
Figure 3. a) IC\textsubscript{50} of SET, SNX, SEA against melanoma cell line; b) Cell morphological observations of B16-F10 cell line incubated with \textit{S. crassifolium} for 24 hours. Black arrows indicate the morphology of cells undergoing apoptosis and different letter indicates the significant different between groups. Methyl palmitate is a major compound found in \textit{S. crassifolium} showed effective cytotoxic roles in B16-F10 cell line. Moreover, the \textit{S. crassifolium} extracted with EA showed a higher cytotoxic activity towards B16-F10 cell line compared to the \textit{S. crassifolium} extracted with ethanol and n-hexane extract with IC\textsubscript{50} (SET IC\textsubscript{50} = 162.30 ± 2.02 μg/mL; SNX IC\textsubscript{50} = 67.10 ± 3.01 μg/mL; SEA IC\textsubscript{50} = 61.26 ± 2.13 μg/mL).

Due to the high amount of methyl palmitate content in the \textit{S. crassifolium} ethyl acetate extract, SEA significantly enhanced anti-cancer effects in B16-F10 cell line as skin cancer cell model. Morphological observation in Figure 3 revealed SEA induces cytotoxic effect by reducing cell density in B16-F10 cells. Moreover, SEA also affected the morphological changes in cell size and shape. These morphological changes can be modulated by methyl palmitate. Methyl palmitate has been reported as a major compound in many medicinal plants. Methyl palmitate has shown cytotoxic potential, antioxidant activity, and anti-inflammatory activity [19-22]. In many other study, methyl palmitate promotes an impactfull decrease in cell viability via inhibition of NF-κB signalling pathway. Likewise, the potential cytotoxic role of methyl palmitate that dominate in SEA are also seen in Figure 4. Hoescht staining was used to determined wether the cytotoxic effect of methyl palmitate was associated with DNA condensation and lead to apoptosis [13][23]. As shown in Figure 4, SEA with the highest methyl palmitate content, was significantly effective to induces apoptotic nuclei (%) on B16-F10 cells. This result indicated that the increasing of methyl palmitate content is positively correlated to apoptosis-induced activity of the extract. However, further analysis related to the molecular and signalling pathway is necessary needed to confirm this result.
4. Conclusion

Based on the multiple variables such as chemical analysis by GC-MS and cytotoxicity potential against B16-F10 cell line, it would be better by extracting the bioactive compounds in *S. crassifolium* using the semi-polar solvent (ethyl acetate) compared to the polar and non-polar solvent. The SEA extract resulted in statistically significant higher chemical constituents and cytotoxicity potential against B16-F10 cell line. The major compound found in the extract is palmitic acid group especially methyl palmitate. Compared to other compounds which are less numerous, methyl palmitate is the compound with the highest probability of being a compound with cytotoxic activity. However, further studies in related to compound purification needs to be done to confirm this study.

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