

LIQUID CHROMATOGRAPHY-
MASS SPECTROMETRY
CHARACTERIZATION OF
BIOACTIVE COMPOUNDS FROM
Sargassum polycystum WITH
THE LARVAL AND IN VITRO
ANTICANCER ACTIVITY

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LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM *Sargassum polycystum* WITH THE LARVAL AND IN VITRO ANTICANCER ACTIVITY

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12 BSTRACT

Sargassum polycystum is a type of seaweed that has been widely used in traditional medicine. This study examines the cytotoxic effect of *S. polycystum* extracts against *A. salina* and the active extract was then fractionated Liquid Vacuum Chromatography (LVC). The fraction was the evaluation of anticancer potential against lung and breast cancer cells in vitro and their chemical components of fractions by LC-MS Bruker micrOTOF-mass spectrometer (ESI -) technique. Ethyl acetate extract was very toxic activity against *A. salina* with LC₅₀ values of 12.37 µg/mL compared to n-hexane with LC₅₀ values of 336.58 µg/mL and methanol with LC₅₀ values of 315.35 µg/mL. The results show that ethyl acetate was obtained from a combined fraction of 4 fractions, these fractions can inhibit lung (H-460 cells) and breast cancer (MCF-7 cells) with concentrations less than 100 µg/mL. Some major components are identified using LC-MS also some components have not yet been identified.

Keywords: *S. polycystum*, MCF-7, H-460, Toxicity, Chemical Identification

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INTRODUCTION

Cancer is still a disease that is a health problem in humans with an increasing number of new patients being diagnosed every year, and the mortality rate is increasing both in men and women especially in developing countries. In 2018, cancer ranked second (9,6 million) the leading cause of death from non-communicable diseases in the global population.¹ High case of cancer and cancer mortality can be caused by risk factors for diet and actions. These factors are high body mass index, lack of consumption of vegetables and fruit, lack of physical activity, smoking and excessive alcohol consumption. In general, 5 types of cancer cause death in the world, namely breast, cervical, colon, lung and stomach cancer.^{2,3}

Among the existing cancers, lung cancer has the highest prevalence in the world. In this case, it is predicted that in 2018, the number of cases of the population in the world who suffer from lung cancer by 2,1 million and will increase every year. Lung cancer treatment is carried out in a various ways including surgery, chemotherapy, and radiotherapy, depending on the stage of the cancer sufferer.⁴ Surgical efforts can be done at an early stage (carcinoma in situ) stage I, and stage II, while chemotherapy can be given at an advanced stage III and several types of postoperative stage II to relieve the risk of recurrence.⁵

Various attempts were made to treat cancer, including using natural products. Natural products are beneficial in the development of anticancer drugs especially those derived from plants. Pharmacological studies show that herbal extracts consist of important nutrients, some compounds that have antitumor, antioxidant and anti-mutation activities with various targets that work synergistically.⁶⁻⁹

Plants that are known to have cytotoxic activity against cancer cells and may be used as anticancer drugs are plants from the family *Phaeophyceae*. *Phaeophyceae* are the most common group of brown algae in Indonesian waters. Several studies have shown that *Phaeophyceae* has the highest antioxidant activity among *Rhodophyceae* and *Chlorophyceae*.^{10,11} One group of brown algae that has currently been

developed is *Sargassum polycystum*. Based on previous research have shown that *S. polycystum* contains bioactive compounds in the form of phenolic, terpenoids, steroids.¹² Other studies have shown that methanol extracts from *S. polycystum* contain steroid compounds, alkaloids, phenolics, flavonoids, steroids, cardiac glycoside, saponins and sterols.¹³ The existence of these chemical components provide bioactivity *S. polycystum* including antioxidants, anti-cancer¹⁴, and UV Protection.^{15,16} Based on this information so that the study conducted an in vitro assessment of the anti-cancer activity of *S. polycystum* on H-460 and MCF-7 cells from various fractions obtained.

EXPERIMENTAL

Sample Extraction

Sample *S. polycystum* was obtained from Dutungan Island, Barru Regency, and South Sulawesi Province, Indonesia. The sample obtained was determined by PAU laboratory, IPB University Indonesia. *S. polycystum* that has been obtained then dried in the oven at a temperature of 40°C. The sample dry obtained was grinded by the grinder. The sample powder was then extracted by multilevel maceration using various solvents, namely n-hexane, ethyl acetate and methanol. *S. polycystum* was macerated with n-hexane solvent 3 times 24 hours and then filtered. The residue was extracted 1 times 24 hours and be strained, and then filtrate evaporated using a rotary evaporator to obtain n-hexane extract. The residue was re-extracted effectively completed with ethyl acetate and methanol in the same way.

Phytochemical Screening

Extracts were determined of the compounds by the colorimetric method using chemical reagents. Tests were carried out with specific reagents based on the method were describes by.^{17,18}

Toxicity Assay by Brine Shrimp Lethality Test (BSLT) Method

The toxicity evaluation was carried out based on the previous methods against brine shrimp lethality dose (BSLT) with slight modification.¹⁹⁻²¹ Data of activity test were analyzed using Lethal Concentration (LC) to determine of concentration the extract could destroy *A. salina* by 50% (LC₅₀).

$$\% \text{ Mortality} = \frac{\text{total of death larvae}}{\text{total of live larvae}}$$

Fractionation of Ethyl Acetate Extract by LVC

The fractionation stage is carried out on *S. polycystum* which has a toxic effect on *A. salina*. Fractionation was carried out on ethyl acetate extract which showed great toxic effects. A total of 10 g of ethyl acetate extract was purified using Liquid Vacuum Chromatography (LVC) and a combination of solvents (n-hexane: EtOAc: MeOH) in various comparisons. Results of the merging of the fractions obtained 4 main fractions (A, B, C, D), which then carried out measurements of LC-MS and anticancer activity test.

LC-MS Analysis of Ethyl Acetate Fraction

Analysis of the chemical content of the *S. polycystum* fraction was carried out using LC-MS Bruker micrOTOF-mass spectrometer (ESI -), with conditions in the column using Cadenza C₁₈ (2x150 mm with flow 0.2 ml/min) following conditions 0-5 min 15-60% Me-CN 0.1%, formic acid, 5-7.5 min 60-70% Me-CN, 0.1% formic acid, and 7.5-10 min 70-80% Me-CN, 0.1% formic acid as mobile phase. Other conditions like nebulizer 0.5 bars, dry heater at 180°C, dry gas at 4.0 l/min were prepared. The injection volume was 0.2 ml per minute at a run time of 15 minutes. The UHPLC was associated with a triple quadrupole mass spectrometer micrOTOF equipped with an electrospray ionization interface that is set in negative mode. Analysis of compounds used software and combination with other literature.

Anticancer Assay of Fraction against of H-460 and MCF-7 by MTT Method

Anticancer assay of each fraction was done using the MTT colorimetric method by *Cancer Chemopreventive Research Center*²² and Dalimunthe *et al*²³, cisplatin was used as positive control and cell of death was calculated with the following formula:

$$\text{Percentage cell of death} = 1 - \left(\frac{\text{Abs sample} - \text{Abs media}}{\text{Abs solvent} - \text{Abs media}} \right) \times 100\%$$

RESULTS AND DISCUSSION

Phytochemical Screening

Phytochemical screening results were carried out from each extract of maceration results using various solvents, hexane, ethyl acetate, and methanol. Phytochemical screening is carried out using specific color reagents. Table-1 shows that n-hexane *S. polycystum* extract contains secondary metabolites in the form of terpenoids, steroids, while the extract of methanol and ethyl acetate *S. polycystum* each contains phenolic metabolites, terpenoids and steroids. Similar results were obtained from the study of Arsianti *et al* which also shows the presence of phenolic compounds, terpenoids, steroids.

Table-1: Phytochemical Screening of *S. polycystum* Extract

<i>S. polycystum</i>	Alkaloid	Flavonoid	Phenol	Saponin	Terpenoid	Steroid
Methanol	-	-	+	-	+	+++
Ethyl acetate	-	+	++	-	+	+++
n-Hexane	-	-	-	-	+	+++

(+) means positive results (++ and +++) means dominant compound, (-) means of negative results.

Toxicity Assay of *S. polycystum* Extract by BSLT Method

Initial screening for bioactivity each extract results were tested for toxicity using the Brine Shrimp Lethality Toxicity (BSLT) method. BSLT testing is an acute toxicity test conducted to determine the toxic effects after dosing within 24 hours. The results of the toxicity test using the BSLT method are express in LC₅₀ value, which indicates the amount of concentration needed to destroy 50% of the larval of *Artemisia salina* shrimp population. The study of Nur *et al* indicated that a sample was declared toxic if it had an LC₅₀ value below 1000 µg/mL.²⁴ The results of the toxicity test of each extract with various concentrations provide an overview of the toxic effects of *A. salina* (Table-2).

Table-2: Toxicity Profile of *S. polycystum* Extract by BSLT Method

Concentration (µg/mL)	Mortality (%)	LC ₅₀ (µg/mL)
Methanol Extract		
200	33.33 ± 5.77	R ² = 0.9493
400	53.33 ± 5.77	LC ₅₀ = 315.35
600	63.33 ± 11.54	
800	76.67 ± 5.77	
1000	83.33 ± 5.77	
Ethyl Acetate Extract		
10	43.33 ± 11.54	R ² = 0.9901
20	66.67 ± 5.77	LC ₅₀ = 12.37
30	76.67 ± 5.77	
40	83.33 ± 11.54	
50	90.00 ± 10.00	
n-hexane Extract		
100	16.67 ± 5.77	R ² = 0.9628
200	26.67 ± 5.77	LC ₅₀ 364.08
300	43.33 ± 11.54	
400	53.33 ± 5.77	
500	63.33 ± 5.77	

Table-2 shows that *S. polycystum* ethyl acetate extract has a very toxic activity with LC₅₀ values 12.37 µg/mL while methanol and n-hexane with LC₅₀ values of 315.35 and 364.08 µg/mL respectively. Based on the results show that the extract of *S. polycystum* was toxic activity because it has an LC₅₀ value <1000 µg/mL.

Characterization by LC-MS Method

Identification of metabolites using LC-MS from various fractions equipped with a micro to-Bruker mass spectrometer with a negative mode. Four fractions of LVC results have been identified using LC-MS analysis and chromatograms can be seen in Fig.-1.

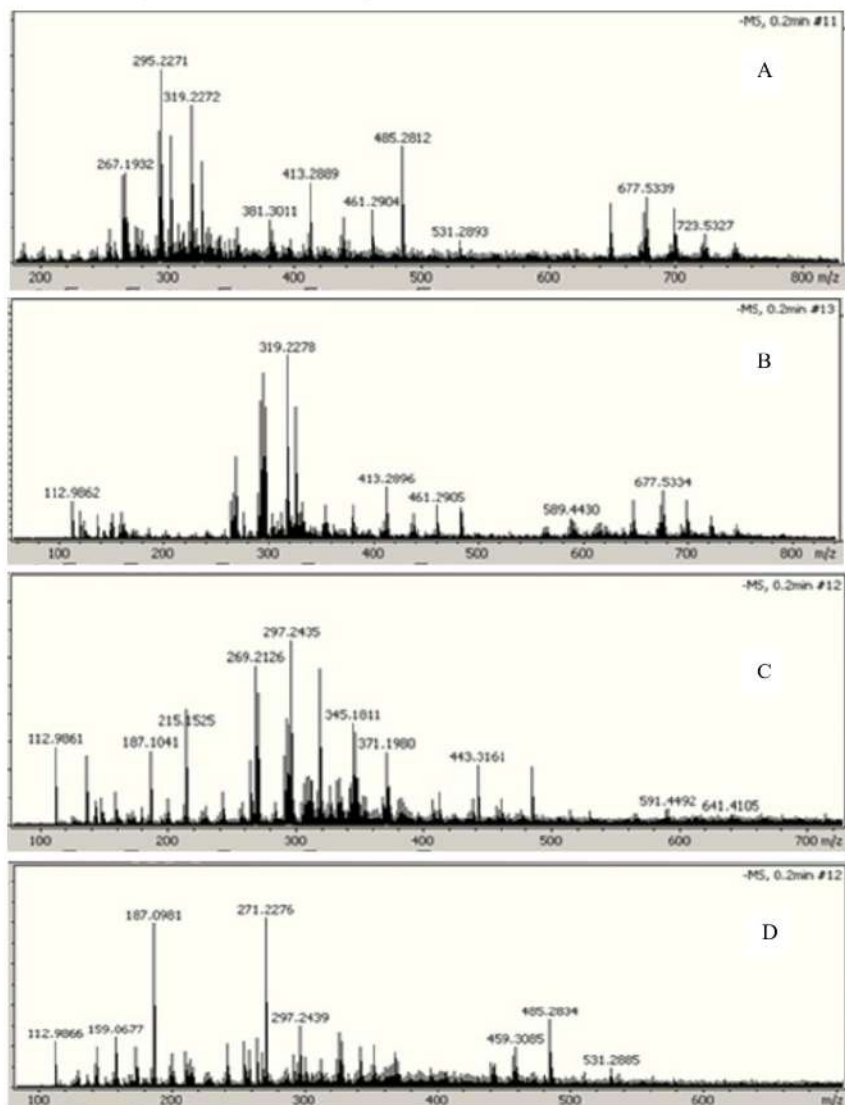


Fig.-1: MS Profile *S. polycystum* Fractions (A, B, C and D) with M-H Mode

Identification of the structure was carried out using literature data on Marine Natural Products Compounds, by the similarity of structural formulas and molecular mass the closest to the MS-spectra. Prediction of compounds can be observed in Table-3.

The data from Table-3 shows some fractions have similar chemical component predictions from each fraction. It is known that in Fraction A, there were 7 predictions of chemical components, Fraction B had

5 chemical components, Fraction C contained 10 types of chemical components, and Fraction D obtained 10 predictions of chemical components. The four fractions generally have similar component compounds, namely 2-Furoic Acid (112 m/z), β -sitosterol, fucosterol (413 m/z). Prediction of compounds in each fraction is thought to be a group of phenolic compounds, flavonoids, steroids and terpenoids. This is similar to the phytochemical screening of *S. polycystum* that contains classes of these compounds.

Table-3: LC-MS Profile of *S. polycystum* Fraction in Negative Mode

Fraction	m/z	Compound	Class
A,B	677	10-carboxyl-pyranopeonidin 3-O-(6"-O-p-coumaroyl)-glucoside	Glycoside Flavonoids
B,C	591	16-oxo-alisol A 24-acetate	Triterpenoid
C,D	531	Phytolaccagenin	Terpenoid
A,D	485	Alisol C	Steroid
B	461	Fucofurodiphloretol	Fenolik
D	459	Quercetin-O-pentoside	Flavonoids
C	443	Thunbergol A, B	Terpenoid
A,B,C,D	413	β -sitosterol, fucosterol	Steroid
A	381	Heptacoxane	Lipid
C	345	[6-ethyl-3,4-dimethyl-(tetrahydro-20,20,60-trimethyl-2H-pyran-30-yl)-2,5-cycloheptadiene]-1-propanoate	Fatty acid
D	327	Ophiopogonanone	Phenol
B	319	p-Coumaroylshicimic acid	Phenol
C,D	297	nd	
A	295	Tanacetol B, Embelin, Phytol	Terpenoid
C	291	Brevifolin carboxylate	Phenol
D	271	Hexadecanoic acid	Lipid
C	269	6,10,14-trimethyl-2-pentadecanoic acid	Lipid
C	215	nd	
D	211	Norisoprenoid derivative	Terpenoid
C,D	187	nd	
A,D	159	nd	
A,B,C,D	112	2-Furoic acid	Phenol

Anticancer Activity against MCF-7 and H-460 Cell

Anti-cancer activity testing of MCF-7 and H-460 cells from each *S. polycystum* fraction was carried out in vitro to describe dead cells using the colorimetric method specifically by MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide} on Microplate Reader with a wavelength of 570 nm. MTT method is a method that can be used to measure the percentage of cell death based on mitochondrial activity from inside a cancer cell.²⁵ The principle of this measurement is based on the formation of color after cells have reacted with MTT reagents. The MTT color which was originally yellow will reduce to purple or blue due to the formation of formazan crystals.

This test carried out using four fractions namely fraction A, B, C, and D. Cytotoxic activity of each fraction is expressed in percentage cytotoxic. The classified cytotoxic activity towards cancer cells shows that IC₅₀ values less than 20 μ g/mL showed strong activity, while IC₅₀ values of 20-100 μ g/mL showed moderate activity and then IC₅₀ values of 100-1000 μ g/mL showed weak activity. Cytotoxic activity testing was carried out with a level series of 10; 100; 1000 μ g/mL. The results showed that each *S. polycystum* fraction provided anticancer activity from strong to moderate. Data on cytotoxic activity of each fraction against MCF-7 and H-460 cells can be observed in Fig.-2.

Figure-2 shows that the fractions C and D in *S. polycystum* with a concentration of 10 μ g/ml gave percent cell death above 50% respectively 70.53 and 71.10% whereas in fractions A and B gave percent cell death below 50% with a value of 42.37 and 22.29%. At a concentration of 100 μ g/ml each fraction was able to give cell death above 50%. Fraction C had the highest cell death activity with a mortality rate of 98.33% followed by fraction B (90.38%), Fraction D (84.4%) and fraction A (54.63%). The results of the

percentage of H460 cancer cell death show that fractions C and D have activities that are categorized as very strong with the level of 10 µg/mL (<20 µg/mL).²⁶

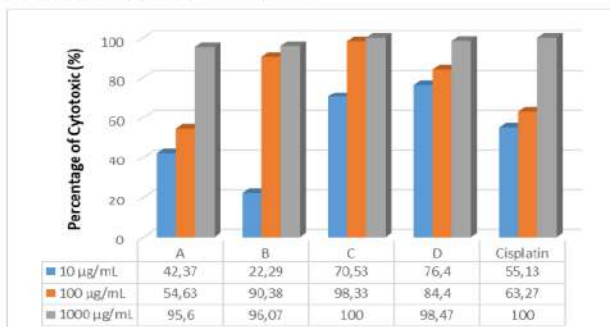


Fig.-2: Anticancer Activity of Fraction of *S. polycystum* against H-460 Cell

The same thing was done for MCF-7 cancer cells using 4 fractions (Fig.-3). The results showed that fractions A, C and D at concentrations of 10 µg/ml gave death activity in MCF-7 cells above 50% with the percentage values of cell death respectively 63.1%, 66.5%, and 67.73% which categorized as having very strong inhibition while fraction B was categorized as a moderate activity where at a concentration of 100 µg/ml gave a percent of cell death of 66.33%. This research shows that fractions C and D provide good cytotoxic activity against H-460 and MCF-7 cells which are categorized as a very strong activity. While fraction A provides cytotoxic activity against MCF-7 cells with a very strong category. Unlike the case with fraction B which only gives cytotoxic to H-460 and MCF-7 cells with the moderate category. With the existence of cytotoxic activity of fractions towards H-460 and MCF-7 cells due to the activity of each component of the compound. Based on the results of the prediction of compounds using LC-MS stated that each fraction contains classes of compounds flavonoids, phenolic, steroids and terpenoids. Flavonoid and phenolic are known to have anti-cancer activity by inducing apoptosis while simultaneously inducing cell differentiation.²⁷⁻²⁹ For groups of steroid/terpenoid compounds can reduce the progression of cancer cells.

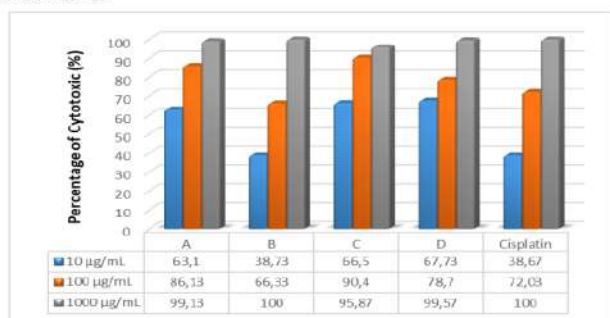


Fig.-3: Anticancer Activity of Fraction of *S. polycystum* against MCF-7

CONCLUSION

Investigations regarding extracts from *S. polycystum* revealed that extract could be developed as a basis for the development of anticancer drugs. The toxicity test for *A. salina* obtained by ethyl acetate extract has a very strong toxic effect. The fractions of ethyl acetate extract have the potential to be anticancer in lung and breast cancer cells in vitro. Also, this study provides an identification of compounds contained in ethyl acetate fraction.

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