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Cytotoxic Test Extract Ethanol Black Sea Cucumber (*Holothuria atra*) Against Line Cells HSC -3 in General *In Vitro*

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Abstract: *Oral Squamous Cell Carcinoma* (OSCC) is one of the most aggressive malignant tumors due to its tendency to spread and high recurrence rate. Sea cucumbers have compounds that show anti- microbial, anti- inflammatory, and anticancer effects. The aim of this study was to determine the anticancer activity extract ethanol black sea cucumber (*Holothuria) atra*) against HSC-3 cell line in vitro. Extract black sea cucumber (*Holothuria) atra*) is done with the type of extraction maceration, then continued with u test Cytotoxicity by MTT *Assay method* using extract ethanol black sea cucumber (Holothuria) atra) at concentrations of 240 µg / ml, 120 µg / ml, 100 µg / ml, 80 µg / ml, 40 µg / ml, 20 µg / ml, 10 µg / ml, 5 µg / ml. At a concentration of 240 µg / ml has a cell viability of 35.215%, doxorubicin has a cell viability of 66.518%. The conclusion of this study is that there is cytotoxicity of ethanol extract of black sea cucumber (*Holothuria atra*) against HSC-3 cell line with IC50 value obtained of 41,371 µ g/ml using ethanol extract of Holothuria atra with concentration of 240 µ g/ml, 120 µ g/ml, 100 µ g/ml, 80 µ g/ml, 40 µ g/ml, 20 µ g/ml, 10 µ g/ml, 5 µ g/ml. At a concentration of 240 µg /ml there is a cell viability of 35,215%, this value is in the moderate toxicity category compared to the positive control of doxorubicin 0.1 µg /ml with a cell viability of 66,518% so that the extract ethanol black sea cucumber (*Holothuria) atra*) has the potential to be an alternative to anticancer drugs.

Keywords*: Oral Squamous Cell Carcinoma (OSCC), Black Sea Cucumber (Holothuria atra), U Test Toxicity, MTT Assay.*

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I. INTRODUCTION

Cancer is a malignant neoplasm caused by uncontrolled cell growth and spread. [1] Abnormal growth of these cells is caused by changes in the expression of several genes. These changes cause the normal cell program to be uncontrolled in the balance of proliferation, differentiation and cell death that supports the growth of the tumor cell population, so that cells will develop into a cell population that can dominate a tissue and spread to other tissues, and can cause death if left untreated [2]. According to the World Health *Organization* (WHO) in 2018, cancer was the second largest cause of death in the world [1].

The most common cancer in the oral cavity is *Oral Squamous Cell Carcinoma* (OSCC), which ranks 16th globally. This cancer is one of the most aggressive malignant tumors due to its tendency to spread and its high recurrence rate. [3]. *Oral Squamous Cell Carcinoma* has a prevalence of 80% to 90% of all malignant neoplasms in the oral cavity [1]. In the oral cavity, OSCC is often found on the edge of the tongue, alveolar mucosa/gingiva and floor of the mouth/ventral tongue [4].

Human Squamous Cell (HSC-3) cell lines are tongue cancer cells that are often used in laboratory research because these cells have the ability to show characteristics similar to the original tumor [5]. Oral cancer usually occurs in men [6]. However, OSCC is also susceptible to adults and the elderly [4].

Oral Squamous Cell Carcinoma has a clinical picture of ulcerated lesions with raised edges around the necrotic center area. Predisposing factors for OSCC are alcohol consumption, smoking, and UV radiation, especially in lip cancer. In addition, there are also other factors associated with this disease, such as exposure to the *Human Papilloma Virus* (HPV), candida infection, and nutritional deficiencies [4]. Treatment of cancer in the oral cavity until now still uses conventional methods such as chemotherapy, radiotherapy, immunotherapy, and surgery. There are several types of drugs used as chemotherapy, but the use of these drugs can cause side effects. Herbal medicine is another active ingredient that can be used as an alternative for new anticancer efforts because its side effects are the least [5]. This herbal medicine can be found in sea cucumbers because

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they contain anti-cancer properties [7]. Sea cucumbers can be found in Indonesian waters [8].

Indonesia is an archipelagic country, the islands in Indonesia are Siberut Island, Sipora, North Pagai and South Pagai Island which are shaped like hills with an area of 6,011.35 km2, which are located in the Mentawai Islands [10]. In Along the coastal waters of the Mentawai Islands there are coral reefs. H animals that live on the shelf reef coral and also on rocky or muddy beaches, one of which is sea cucumbers [10]. Sea cucumbers live from shallow areas to deeper waters, but sea cucumbers prefer to live in waters free from pollution, and relatively calm water [9]. Sea cucumbers are one of the animals with high economic value and are exploited commercially in tropical areas [10].

Sea cucumbers or *Holothuroidea* are marine organisms that are classified as phylum *Echinodermata* [11]. Several types of *Holothuroidea* is *Holothuria scabra, Holothuria atra, Stichopus veriegatus,* and *Stichopus hermanni* [12]. Sea cucumbers are soft organisms that are elongated like worms. One of the invertebrate organisms unique, has a flexible cylindrical body, rough skin, and has fine tentacles that function to eat microscopic algae in order to absorb nutrients from marine organic matter [13].

Nutritional composition Sea cucumbers have many health benefits, including preventing or treating various diseases. [14]. One of the nutritional compositions is antioxidants, antioxidants are one of the compounds found in sea cucumbers which can reduce oxidative stress caused by free radicals. [14]. Sea cucumbers are marine animals rich in novel biologically active molecules, including compounds that exhibit anti- microbial, anti- inflammatory, and anticancer effects [15].

Research that has been conducted by Halimatushadyah *et al.* (2018) showed that ethanol extract of sea cucumber with a concentration of 20 µg/ml can induce apoptosis by 82.06%. [16] In addition, there are also previous research results showing anticancer activity in black sea cucumber content with an *inhibition concentration* 50 (IC_{50}) value for maceration extract toxicity of 81.0153 ± 4.51 g/ml, while the IC 50 value for anticancer activity possessed by black sea cucumber is 0.0404 ± 0.004 g/ml, 0.00. All of these findings indicate high cytotoxicity. The test was carried out using the *Microculture method Tetrazolium Technique Assay* (MTT *Assay*) [14].

The MTT method is a sensitive, quantitative, and reliable colorimetric method for measuring cell viability, proliferation, and activity. [17]. Based on this background, researchers are interested in conducting research with ethanol extract of black sea cucumber (*Holothuria atra*) to determine the cytotoxic activity on HSC-3 cell lines *in vitro* with cytotoxic testing using the MTT test method. The purpose of this study was to determine the anticancer activity of ethanol extract of black sea cucumber (*Holothuria atra*) on HSC-3 cell lines in *vitro.*

II. MATERIALS AND METHODS

This research is planned from October 2024 - December 2024. The type of research conducted is an *in vitro laboratory experimental study*. The research design used is a cytotoxicity test with a *post-test only control group design plan.* The population in this study was black sea cucumbers (*Holothuria atra*) obtained in the Mentawai Islands and HSC-3 cell lines derived from *Oral Squamous Cell Carcinoma patients.* The samples in this study were black sea cucumbers (*Holothuria atra*) obtained in a wet state and HSC-3 cell lines obtained from the Integrated Laboratory of Yarsi University, Jakarta. The number of samples used in this study was 33 The samples were divided into 11 groups, each group consisting of 3 repetitions.

The samples used will be divided into test groups, namely:

- Group (+) : Positive control (Doxorubicin 0.1 µg /mL)
- Group (-) : Negative control (DMSO)
- *Blank* Group : HSC-3 Cells
- Group 1: Sea cucumber extract with a concentration of 240 µg /mL
- Group 2: Sea cucumber extract with a concentration of 120 µg /mL
- Group 3: Sea cucumber extract with a concentration of 100 µg /mL
- Group 4: Sea cucumber extract with a concentration of 80 µg /mL
- Group 5: Sea cucumber extract with a concentration of 40 µg /mL
- Group 6: Sea cucumber extract with a concentration of 20 µg /mL
- Group 7: Sea cucumber extract with a concentration of 10 µg /mL
- Group 8: Sea cucumber extract with a concentration of 5 µg /mL

In a study conducted at the Faculty of Dentistry, Baiturrahmah University, Padang, a series of experiments were carried out to test cytotoxicity. extract ethanol from black sea cucumber (Holothuria atra). This study aims to observe the effect of the extract on the viability of HSC-3 cells obtained from Yarsi University.

The research process begins with the preparation of the tools and materials used. Tools such as digital scales, calipers, dark bottles, glass funnels, test tubes, autoclaves, and CO2 incubators are carefully prepared. Meanwhile, the main materials used include 2 kg of fresh black sea cucumbers, ethanol as a solvent, and cell culture media such as DMEM and FBS.

Extraction black sea cucumber is done by maceration method. Sea cucumber samples that have been cleaned and cut into small pieces are soaked in ethanol for three days, with routine stirring every day. After that, the soaking solution filtered using filter paper and evaporated using a rotary evaporator at 40 °C until a clear, thick extract is obtained.

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Next, HSC-3 cells were cultured in DMEM medium enriched with FBS and antibiotics. These cells were placed in a CO2 incubator at 37°C to stay alive and grow. After culturing, cell counts were performed using a hemocytometer. Cells that had been mixed with trypan blue solution were counted under a microscope to ensure proper cell density before testing.

Cytotoxicity test was performed using the MTT Assay method. HSC-3 cells were placed in a 96-well microplate and treated with various concentrations of extract. black sea cucumber, ranging from 5 to 240 µg/ mL. After 24 hours of incubation, the medium was replaced with MTT solution to observe changes in cell viability. Then, DMSO solution was added to dissolve crystal formazan formed. Absorbance measurements were performed using a microplate reader at a wavelength of 570 nm.

The results obtained were analyzed to determine the IC50 value, which is the concentration of extract that causes the death of 50% of test cells. The higher the IC50 value, the lower the level of toxicity. extract on cells. From the results of this study, it is hoped that information will be obtained regarding the potential of the extract ethanol black sea cucumber in the development of cancer therapy or other medical applications.

III. RESULTS

A. Results of Making Black Sea Cucumber Extract (Holothuria Atra)

The initial process of this research was carried out by making black sea cucumber extract using the maceration method to obtain clear results which were then evaporated to form a thick extract weighing 700 grams from 2 kg of wet sea cucumber.

B. Cell Culture Results

HSC-3 cells brought from Yarsi University were cultured in DMEM containing 10% FBS and mixed with 100 µg/mL streptomycin and 100 units/mL penicillin (antibiotic-antimocotic) which were subcultured at a temperature of 37% in a 5% CO2 incubator. Cells that are ready to be used will be counted first using a microscope with 10x objective magnification (Figure 1).

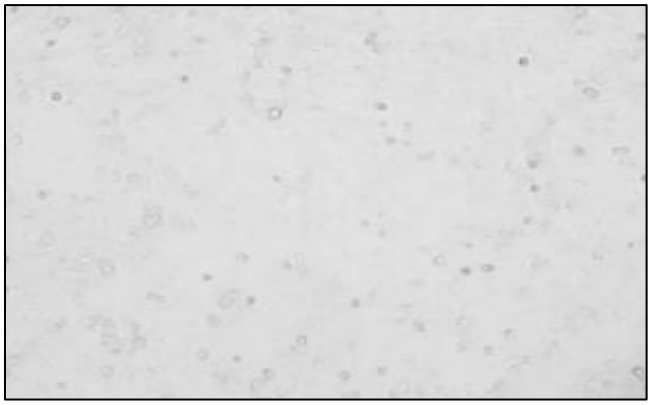

Fig 1 Personal Documentation of HSC-3 Cell Culture with 100x Magnification.

C. Research Results of Cytotoxicity Test of Black Sea Cucumber Extract

Research that has been conducted to determine the cytotoxic activity of ethanol extract of black sea cucumber (*Holothuria atra*) on HSC-3 cell lines *in vitro* obtained the following results:

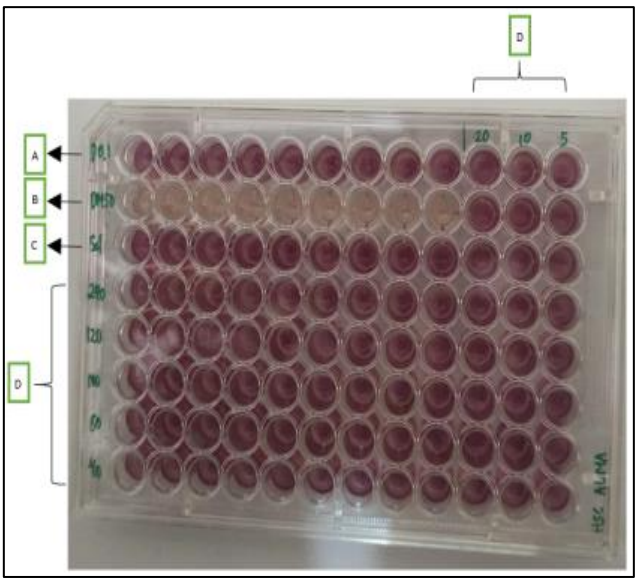

Fig 2. Cytotoxicity Test a). Doxorubicin, b). DMSO, c). Cells, d). Concentration (240, 120, 100, 80, 40, 20, 10, 5) µ g/Ml.

The image above (image 2) is the result of a cytotoxic test of the ethanol extract of black sea cucumber (Holothuria atra) on the HSC-3 cell line using a concentration of 240 µ g/mL, 120 µ g/mL, 100 µ g/mL, 80 µ g/mL, 40 µ g/mL, 20 µ g/mL, 10 µ g/mL, 5 µ g/mL, positive control and negative control marked by the appearance of formazan crystals formed. Formzan crystals that are dark purple indicate the number of living cells, while formazan crystals that are colorless or that appear yellow indicate dead cells. The results of viability measurements from absorbance data formed in each concentration using spectrophotometry at a length of 570 nm are as follows:

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Table 1. Sample Absorbance and Viability Results Data

	Repetition			Average	Via mobility
	1	2	3		
240	0. 451	0. 385	0. 369	0.481	35. 215
120	0. 413	0. 370	0. 447	0.621	36. 454
100	0. 388	0.425	0.431	0.626	37.147
80	0.443	0.452	0.411	0.671	40.218
40	0.455	0.681	0.477	0.682	55.423
20	0.623	0.548	0.517	0.689	59.138
10	0.525	0.773	0.539	0.695	66.518
5	0.540	0.573	0.777	0.712	69.143
sel	0.879	0.800	0.834	0. 8 0 7	
media	0.1 56	0.1 65	0.1 7 3	0.1 42	
doxorubicin	0.602	0.613	0.622	0.612	66,518

The table above shows that the highest viability of HSC-3 cell lines was obtained from the ethanol extract of black sea cucumber at a concentration of 5 µ g/mL has a viability of 69.143 %, while at a concentration of 240 µ g/mL has a cell viability of 35.215 % with a low viability category, in the positive control of doxorubicin there is a cell viability of 66.518%. The results of cell viability indicate that the higher the viability value, the more living cells. From these data it can be seen that a concentration of 240 µ g/mL can provide benefits that show fewer living cells compared to doxorubicin.

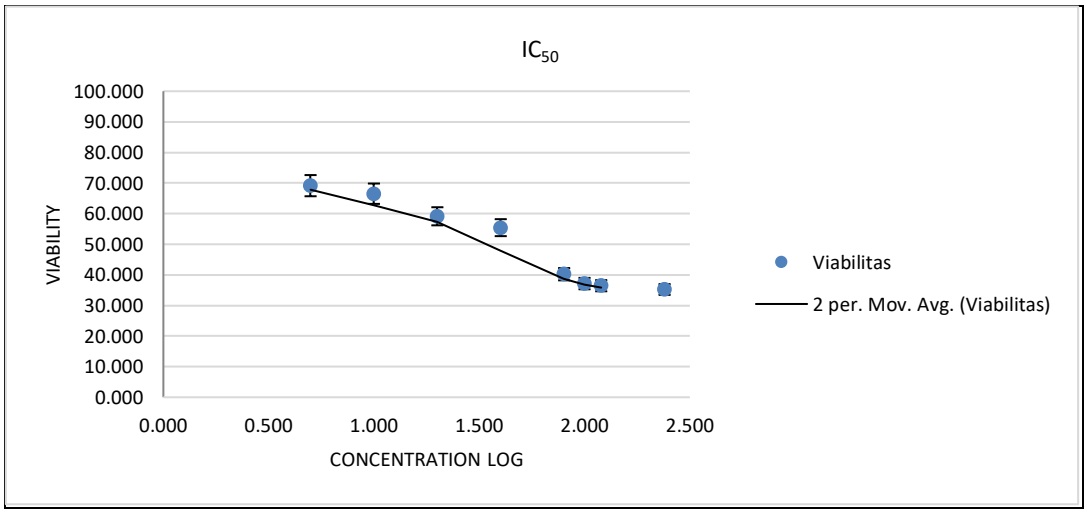

Fig 3. Graph of the Relationship between Log Concentration and Cell Viability

Graphic on Linear regression was performed to obtain the IC50 value using concentration to determine whether the ethanol extract of black sea cucumber is toxic to HSC-3 cell lines. The results were entered into the linear regression equation of quercetin with y = -23.848x + 88.555, with coefficient correlation R2 = 0.9437. After being calculated, the total $_{IC50}$ value of the preparation was obtained at a concentration of 240 µ g/mL, 120 µ g/mL, 100 µ g/mL, 80 µ g/mL, 40 µ g/mL, 20 µ g/mL, 10 µ g/mL, 5 µ g/mL.

Table 1. IC $_{50}$ Result Data

Concentration (µ g/mL)	Viability	IC $_{50}$	Description
240	35. 215	41,371	moderate toxicity
120	36. 454		
100	37. 147		
80	40. 218		
40	55,423		
20	59,138		
10	66. 518		
5	69.143		

The table above is the result of IC $_{50}$ to determine that the preparation The ethanol extract of black sea cucumber (*Holothuria atra*) is toxic. This cytotoxicity test was carried out with 3 repetitions using *the microtetazolium* (MTT) method on HSC-3 cell lines. Overall, the cytotoxic activity was in the moderate cytotoxicity category with an IC $_{50}$ value 41,371 µ g/mL in extract ethanol *Holothuria atra* with a concentration of 240 µ g/mL, 120 µ g/mL, 100 µ g/mL, 80 µ g/mL, 40 µ g/mL, 20 µ g/mL, 10 µ g/mL, 5 µ g/mL.

IV. DISCUSSION

The results of the study showed that there was cytotoxicity of ethanol extract of black sea cucumber *(Holothuria atra)* against HSC-3 cell lines. in concentration 240 µ g/mL. According to research conducted by Yuliana *et al.*, 20 22, regarding the anti- cancer cytotoxic test extract ethanol *Holothuria atra* against larvae A. salina The research results showed that the extract ethanol *Holothuria Atra* contains secondary metabolites which can influence activity against larvae A. salina because of existence compound holothurin from sea cucumber *Holothuria the atra* that nature

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toxic with mark <30ppm. [20] These findings support previous research, namely the formation of antioxidant activity in the strong category. with an IC_{50} value of 33.514 mg/mL in the extract ethanol *Holothuria atra* with concentrations of 10 ppm, 30ppm, 50ppm, 70ppm, 90ppm [21].

Other research conducted by Misgiati *et al.*, 2024 showed anticancer activity in black sea cucumber content with an *inhibition concentration* 50 (IC_{50}) value for maceration extract toxicity of 81.0153 ± 4.51 g/ml, while the IC 50 value for anticancer activity possessed by black sea cucumber was 0.0404 ± 0.004 g/ml. [14] Another study using sea cucumber species Holothuria sp carried out by Arma., 2018, there are active triterpene compounds glycoside sea cucumber arise underpass *(Holothuria atra)* has a very strong anticancer activity (IC_{50} = 0.23 µg / mL) against Supri's Clone 1 Cells (Sp-C1). [22] The ability of ethanol extract of black sea cucumber *(Holothuria atra)* in the toxicity effect In this study, when compared with previous studies, it can be seen that the concentration level used was higher, and the types and properties of the test cells were also different.

The increasing concentration of black sea cucumber extract gives a greater toxicity effect because the more extract is given, the higher the impact on cell death [23]. In addition, the condition or state of the sea cucumber also affects its overall content, fresh sea cucumbers have a protein content of 7.5%, fat 3.09%, water 69.52%, ash 3.5%, and carbohydrates 16.39% [24].

Based on the research that has been done, it was found that the results of the cytotoxic test showed that the higher the concentration, the lower the cell viability. The concentration of extract that can kill cells is 50.926%, namely at a concentration of 240 µg/mL compared to a concentration of 10 µ g/mL and 5 µ g/mL. The active compounds in sea cucumber extract can trigger greater oxidative reactions, contributing to cancer cell damage and increasing cytotoxicity. With increasing concentration of the extract, the ability to induce apoptosis in cancer cells also increases, making the extract Black sea cucumber as a potential candidate in therapy anticancer.[25]

The positive control in this study used doxorubicin 0.1 µ g/mL. The mechanism of action of doxorubicin involves inhibition topoisomerase II enzyme which is important for the DNA replication process in cancer cells. By blocking this enzyme, doxorubicin prevents cancer cells from dividing and proliferating, thereby causing cancer cell death. [26]. Doxorubicin 0.1 µg /mL can affect cell viability. Dox orubi c in produces free radicals, which can damage cell membranes and DNA. In addition, dox orubi c in produces free radicals, which can damage cell membranes and DNA. After entering the cell through passive diffusion, doxorubicin accumulates within cells, especially in the compartment nucleus. However, doxorubicin causes toxicity to the heart, brain, liver, and kidneys because it is not selective for cancer cells. [27]. The use of DMSO is used in negative control so that bias caused during research can be minimized and in the dilution of treatment extracts is better [28].

The initial process of this research was carried out by making ethanol extract. black sea cucumber (*Holothuria) atra*). Preparation of preparations the dose is made 8 with a concentration 240 µ g/mL, 120 µ g/mL, 100 µ g/mL, 80 µ g/mL, 40 µ g/mL, 20 µ g/mL, 10 µ g/mL, 5 µ g/mL. Extract sea cucumber black *(Holothuria atra)* used in this study was made by maceration method. Extraction method is important to obtain active substances from a material. The maceration process is very beneficial in extracting natural compounds, because by soaking, the solvent will have a longer interaction time with the sample to break down the walls and cell membranes of the sample.

Based on the results of the research that has been carried out, it can be concluded that there is cytotoxicity of ethanol extract of black sea cucumber *(Holothuria atra)* against HSC-3 cell lines. with the IC50 value obtained of 41,371 µ g/mL using *Holothuria atra* ethanol extract with a concentration of 240 µ g/mL, 120 µ g/mL, 100 µ g/mL, 80 µ g/mL, 40 µ g/mL, 20 µ g/mL, 10 µ g/mL, 5 µ g/mL. At a concentration of 240 µ g/mL there was a cell viability of 35.215 %, this value is in the moderate toxicity category compared to the positive control of doxorubicin 0.1 µ g/mL with a cell viability of 66.518%.

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