CYTOTOXIC TEST OF BIOACTIVE COMPOUNDS FROM FRUIT BODY EXTRACTS OF *Pleurotus ostreatus* ON BREAST CANCER CELLS

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Abstract. The purpose of this study was to identify the classes of bioactive compounds in the n-hexane, chloroform, and ethyl acetate extracts from the fruiting body of the Pleurotus ostreatus mushroom, as well as to evaluate the cytotoxic activity of these extracts against T47D and MCF7 breast cancer cells. The methods employed were both descriptive and experimental. The independent variable in this study was the administration of P. ostreatus extract to cancer cell cultures at various concentrations (1000, 500, 250, 125, 62.50, and 31.25 µg/mL). The MTT assay was used to measure the cytotoxic activity of the mushroom extracts against T47D and MCF7 breast cancer cells. The main parameters analyzed included the percentage of cell viability, the IC_{50} value, and the types of bioactive compounds based on the KLT test. Data were analyzed using linear regression, while the detection of bioactive compounds was conducted descriptively by observing the eluted color spots. The results indicated that the n-hexane, chloroform, and ethyl acetate extracts of P. ostreatus contained bioactive compounds belonging to the alkaloid, flavonoid, and terpenoid groups. The n-hexane extract exhibited cytotoxic effects against T47D cells with an IC_{50} value of 990.78 µg/mL and against MCF7 cells with an IC_{50} value of 555.22 µg/mL. The chloroform extract demonstrated cytotoxic effects against T47D cells with an IC_{50} value of 21.564 µg/mL and against MCF7 cells with an IC_{50} value of 371.15 µg/mL. The ethyl acetate extract showed cytotoxic effects against T47D cells with an IC_{50} value of 3.226 µg/mL and against MCF7 cells with an IC50 value of 371.15 µg/mL.

Keywords: MCF7 breast cancer cells, MTT assay, *Pleurotus ostreatus*, T47D breast cancer cells, TCL

A. Introduction

Edible mushrooms are utilized by the wider community as a food source and source of medicine because they have high nutritional value and contain several bioactive compounds that are beneficial to health. According [1], *P. ostreatus* mushrooms can produce bioactive compounds such as alkaloids, flavonoids, and terpenoids, and have many benefits such as anti-inflammatory, antimicrobial, antioxidant, and anticancer. The content of bioactive compounds in *P. ostreatus* mushrooms as anticancer supports this research.

Cancer is one of the many deadly diseases that can occur in humans regardless of age, gender or race. Data from the Ministry of Health reports that around 6% or 13.2 million Indonesians suffer from cancer and the disease is the fifth leading cause of death in Indonesia [2]. Breast cancer is the most common cancer diagnosed in women and is the second leading cause of death in women worldwide. Data from the Ministry of Health reports that the number of breast cancer cases in Indonesia reaches 42.1 per 100,000 women. Based on data from the

Global Burner of Cancer (GLOBOCAN) in 2020, it was reported that breast cancer was the worst case in women with 2.3 million new diagnoses and 684,996 deaths in women worldwide [3].

Causes of breast cancer include aging, family history of breast cancer, late menopause, low parity, hormonal influences, excessive alcohol consumption, and excessive dietary fat intake. Breast cancer cells have several types to be studied, including T47D (Human Ductal Breast Epithelial) and MCF7 (Michigan Cancer Foundation-7) cancer cells, both of which are grouped into the luminal A subtype. Common treatments for breast cancer patients are surgery, chemotherapy, and radiotherapy. Doxorubicin is an anthracycline chemotherapy drug used in the treatment of breast cancer. Doxorubicin works by binding to the DNA of cancer cells, so that cancer cells cannot grow. [4], [5].

The effects of using chemotherapy drugs can have an impact on the health of the body, because it not only kills cancer cells, but can also attack normal cells. One solution that can be done is to utilize various bioactive compounds found in nature. Bioactive compounds such as anticancer derived from *P. ostreatus* mushroom extract have the ability to effectively handle cases and reduce side effects that occur. The purpose of this study was to determine the bioactive compounds of n-hexane, chloroform, and ethyl acetate extracts of *P. ostreatus* mushroom fruiting bodies and to determine the cytotoxic activity of n-hexane, chloroform, and ethyl acetate extracts against T47D and MCF7 breast cancer cells. This research is expected to reveal bioactive compounds that have potential as breast anticancer.

B. Methods

This research was conducted at the Mycology and Phytopathology Laboratory of the Faculty of Biology, Jenderal Soedirman University for the extraction stage of *P. ostreatus* mushrooms. The KLT test was conducted at the Pharmaceutical Biology Laboratory, Faculty of Pharmacy, Muhammadiyah Purwokerto University. The cytotoxic activity test of *P. ostreatus* mushroom extract against T47D and MCF7 breast cancer cells was conducted at the Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University. The research was conducted from April to September 2024.

The materials used in the study include *P. ostreatus* mushroom simplicia, aluminum foil, plastic wrap, latex gloves, masks, filter paper, tissue paper, label paper, silica gel F254, n-hexane solvent (PA), chloroform solvent (PA), ethyl acetate solvent (PA), vanillin-sulfuric acid reagent, AlCl3 reagent, Dragendorff reagent, glacial acetic acid, toluene, formic acid, aquabidest, trypsin, NaHCO3, NaOH, HEPES, 10% FBS, 1% penicillin-streptomycin antibiotics, 1% fungicide, 10% SDS, 0.1 N HCl, MTT, Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) medium, and T47D and MCF7 cell cultures.

Tools used in the study include Erlenmeyer flask, 1000 mL and 500 mL beaker glass, 500 mL measuring cup, balance, 500 mL duran bottle, vial bottle, porcelain cup, hot plate, 96 well microplate, vacuum pump, rotary evaporator, oven, KLT chamber, centrifuge, haemocytometer, stirring rod, capillary tube, spoon, sprayer, Buchner flask, magnetic stirrer, inverted microscope, refrigerator, incubator, ELISA reader, laboratory coat, and camera.

This research was conducted using descriptive and experimental methods. The research stages consisted of extraction, detection of bioactive compounds, and cytotoxic activity test of *P. ostreatus* fruiting body extract against T47D and MCF7 breast cancer cells. The cytotoxic test treatment was carried out by giving n-hexane, chloroform, and ethyl acetate extracts with different concentrations (1000, 500, 250, 125, 62.50, 31.25) µg/mL to each T47D and MCF7 breast cancer cell culture, the treatment was carried out as many as 3 replicates for each concentration of extract tested. MTT assay was performed to determine the cytotoxic activity of *P. ostreatus* fruiting body extract against T47D and MCF7 breast cancer cells. The

independent variable in this study was the administration of P. ostreatus fruiting body extract to T47D and MCF7 breast cancer cell cultures with different concentrations (1000; 500; 250; 125; 62.50; 31.25) µg/mL, while the dependent variable in this study was the inhibition of cancer cell growth. The research parameters were the percentage of cell viability, IC_{50} value, and compound groups based on the KLT test.

C. Results And Discussion

Extraction of *P. ostreatus* fruiting bodies was carried out by multistage maceration method using 3 types of solvents with different levels of solubility, namely n-hexane, chloroform, and ethyl acetate. The level of solubility in each solvent will affect the amount of bioactive compounds extracted. The yield value serves to determine the level of secondary metabolites carried by the solvent. The yield of *P. ostreatus* fruiting body extract based on the type of solvent used in the maceration process produces a different percentage yield value. The results of n-hexane, chloroform, and ethyl acetate extracts of *P. ostreatus* mushroom fruiting bodies obtained by the multistage maceration method weigh 0.71 g, 1.43 g, and 0.68 g, respectively, from a dry powder weight of 150 g with a yield value of 0.47%, 0.95%, and 0.45%.

Based on the yield value, it is known that the percentage of chloroform extract is higher than that of n-hexane extract and ethyl acetate extract. This shows that more non-polar bioactive compounds are extracted compared to semi-polar bioactive compounds. According to [6] the difference in solvent polarity used will produce different values, because during the extraction process the solvent diffuses to components that have the same level of polarity.

The difference in percentage yield value in the extract shows the number of bioactive compounds contained in it. According to), the yield value is related to the number of bioactive compounds contained in a sample. The higher the yield value of an extract, the higher the content of bioactive compounds interested in the sample [7]. Factors that can affect the yield of extracts from a solvent are the type of solvent used, the extraction method used, the length of extraction time, the particle size of a material, and the ratio of the number of samples to the amount of solvent. [8].

Table 1. Detection of Bioactive Compounds of *P. ostreatus* Extracts

P. ostreatus extract	Dragendorff reagent	$AlCl_3$ Reagent	Vanillin-Sulfuric Acid Reagent
	Alkaloids Yellow/Orange	Terpenoids Purple-black	Flavonoids Yellow/Blue Green
n-hexane	+	+	+
Chloroform	+	+	+
Ethyl Acetate	-	+	+

Notes: Compound detected (+), compound not detected (-)

Based on Table 1. the detection results of bioactive compounds of n-hexane and chloroform extracts of *P. ostreatus* mushrooms contain bioactive compounds of alkaloid, terpenoid, and flavonoid groups. The ethyl acetate extract of *P. ostreatus* was detected to contain terpenoids and flavonoids, but no alkaloid compounds were detected. This is thought to occur due to differences in polarity between alkaloid compounds and the solvents used. Based on the results of the KLT test of *P. ostreatus* mushroom extract, this is in accordance with research conducted by [9] *P. ostreatus* mushrooms contain bioactive compounds of alkaloid, flavonoid and terpenoid groups.

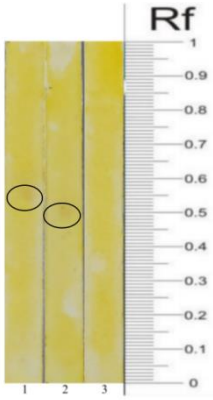

Figure 1. Chromatogram of alkaloid test

Description: (1) chloroform extract; (2) n-hexane extract; (3) ethyl acetate extract.

Figure 1 shows that the n-hexane extract and chloroform extract of *P. ostreatus* mushrooms contain alkaloid compounds characterized by brownish yellow spots after spraying the KLT plate with Dragendorff reagent. Detection of alkaloid compounds in ethyl acetate extract did not show any brownish yellow spots. According to [10] alkaloid compounds will produce brownish yellow to orange spots when reacting with Dragendorff reagent. In this reaction there is a ligand replacement in which nitrogen which has a free electron pair on the alkaloid forms a coordinate covalent bond with the K + ion of potassium tetraiodo bismutat producing a potassium-alkaloid complex. In the KLT test there is an Rf value indicated by a color spot that elutes on the KLT plate.

Detection of flavonoid bioactive compounds of *P. ostreatus* mushroom extracts was carried out by KLT test and the results were viewed with UV light at a wavelength of 366 nm. The detection results of flavonoid compounds in n-hexane, chloroform, and ethyl acetate extracts can be seen in the following figure:

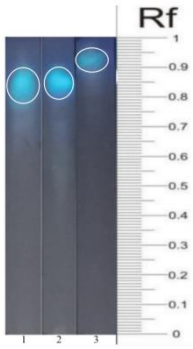

Figure 2. Chromatogram of flavonoid test

Description: (1) n-hexane extract; (2) chloroform extract; (3) ethyl acetate extract

Figure 2. shows that the n-hexane, chloroform, and ethyl acetate extracts of *P. ostreatus* mushrooms contain flavonoid bioactive compounds characterized by the presence of greenish-blue spots on the KLT plate. The flavonoid test will show a blue to purple spot when viewed under UV light with a wavelength of 366 nm. According to [11], the mechanism of spot

appearance of flavonoid compounds in UV light 366 nm is that the spot will fluoresce and the KLT plate will appear dark because there is an interaction between UV light and the chromophore bound by the auxochrome in the spot. The Rf value of Flavonoid Group Bioactive Compounds from n-hexane 0.83; chloroform 0.79; and ethyl acetate 0.92.

Detection of terpenoid group bioactive compounds of n-hexane, chloroform, and ethyl acetate extracts of *P. ostreatus* mushrooms was carried out by KLT test. The detection results of terpenoid compounds can be seen in the following figure:

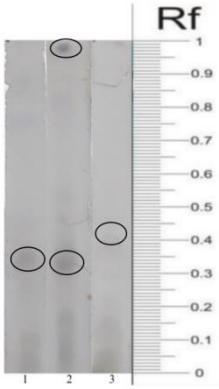

Figure 3. Terpenoid test chromatogram

Description: (1) n-hexane extract; (2) chloroform extract; (3) ethyl acetate extract

Figure 3 shows that the n-hexane, chloroform, and ethyl acetate extracts of *P. ostreatus* mushrooms contain terpenoid bioactive compounds characterized by the presence of a purple-black spot. In the n-hexane extract, the spot eluted more strongly than the chloroform and ethyl acetate extracts which eluted weakly. Terpenoid compounds can be detected with vanillin-sulfuric acid reagent with the mechanism of forming compounds that have conjugated double bonds. The double bond in the terpenoid chemical structure has an absorption spectrum in ultraviolet light and visible light, so detection in the visible light area looks violet in color. The Rf values of terpenoid bioactive compounds from n-hexane extract are 0.32 and 0.97; chloroform extract 0.34; ethyl acetate extract 0.38. According to [12], the highest Rf value of terpenoid compounds is 0.92 cm and tends to be distributed on non-polar mobile phases. According to research conducted by [13], that the Rf value of terpenoid compounds of S. commune mushroom extract ranges from 0.13 - 0.94 cm. Triterpenoids are terpenoid derivative secondary metabolites that have benefits as anti-inflammatory and anticancer.

The cytotoxic test of n-hexane, chloroform, and ethyl acetate extracts of *P. ostreatus* against T47D and MCF7 breast cancer cells was carried out using MTT assay. The results of the MTT assay showed that the mitochondria of cancer cells that are still alive will absorb MTT reagent so that the reduction reaction of tetrazolium salt which is initially yellow into purple formazan crystals occurs. In cancer cells that have died, formazan crystals do not form due to mitochondrial metabolism being stopped so that they remain yellow. According to [14], MTT reagent is a mono-tetrazolium salt consisting of a positively charged quaternary tetrazole ring containing 4 hydrogen atoms and surrounded by 3 aromatic rings, namely two phenyl groups and one thiazolyl ring. MTT reduction will damage the core of the tetrazole ring and form a purple molecule insoluble in water called formazan crystals. MTT reagents can pass through cell membranes and membranes in the mitochondria of living cells so that the reduction of tetrazolium salts into formazan crystals occurs.

The IC_{50} value indicates the concentration value that causes 50% inhibition of cell proliferation and indicates the toxicity of a compound to cells. As the concentration of *P.*

ostreatus mushroom extract increases against T47D and MCF7 cancer cells, cancer cell viability decreases. According to [15], toxicity is a condition where a material has a toxic effect. A compound is said to be toxic if the compound causes toxic effects within 24 hours. According to [16], differences in cell viability can be caused by differences in interactions between cell proteins and bioactive compounds and differences in extract doses. The higher the dose of extract used, the higher the bioactive compounds that can result in higher cell death. The IC_{50} value of the Cytotoxic Test of *P. ostreatus* Mushroom Extract can be seen in Table 2.

Tabel 2. IC_{50} Value of Cytotoxic Test of *P. ostreatus* Extract

P. ostreatus extract	**IC_{50} Value of Cancer Cells (µg/mL)**	
	T47D cells	**MCF7 cells**
N-hexane	990.78	555.22
Chloroform	21.564	371.15
Ethyl Acetate	3.226	397.88

Based on Table 2., there are differences in IC_{50} values after the administration of *P. ostreatus* mushroom extracts against T47D and MCF7 cancer cells tested. The IC_{50} value of ethyl acetate extract against T47D cancer cells has a lower IC_{50} value of 3.226 µg/mL, chloroform extract of 21.564 µg/mL, and n-hexane extract of 990.78 µg/mL. In MCF7 cancer cells, chloroform extract has a lower IC_{50} value of 371.15 µg/mL, ethyl acetate extract of 397.88 µg/mL, and n-hexane extract of 555.22 µg/mL. Based on the IC_{50} value, it shows that the ethyl acetate extract has very strong toxicity and chloroform extract has strong toxicity to T47D cells, while in MCF7 cells, *P. ostreatus* mushroom extract has weak toxicity.

Cancer cells can experience changes in cell morphology due to exposure to active compounds which is a reflection of biochemical conditions that lead to cell death by apoptosis or necrosis. Cancer cells that are still alive have an elliptical morphology resembling leaves, while cancer cells that experience death have a morphological change to be damaged in a round shape and appear wrinkled. According to [17], cell shrinkage and changes in cell shape to round are signs of cancer cells undergoing apoptosis.

T47D cells are a type of cancer cell that has mutations in the p53 gene, so that the p53 gene cannot bind to DNA and reduces the ability to regulate the cell cycle and spur apoptosis. The occurrence of mutations in the p53 gene results in gene damage so that apoptosis does not occur and damaged cells replicate which eventually become cancer cells [18]. In MCF7 cells, the p53 gene has not been mutated, overexpression of Bcl-2, and does not express caspase-3 so that it can avoid apoptosis [16]. The p53 gene plays a role in controlling the cell cycle and DNA replication. If there is damage to the DNA, the p53 gene will restrain the cell from entering the next phase and induce enzymes to repair the DNA. If DNA damage cannot be repaired, the p53 gene will direct the cell to die by apoptosis. Caspases are cysteine proteases that play a role in apoptosis. Caspase 3 is one of the caspases that plays a role in the induction of apoptosis which is activated through intrinsic or extrinsic pathways by caspase-8 and caspase-9. Caspase-3 acts as an executor caspase that performs apoptosis on damaged cells. Apoptosis is programmed cell death. Cell death due to apoptosis is characterized by DNA fragmentation in the nucleus, condensation of chromatin material, cell shrinkage, and loss of extracellular matrix adhesion. According to [19], in apoptosis there are two signaling pathways, namely cytotoxicity through the intrinsic pathway (mitochondria) and an increase in death ligands on death receptors in the extrinsic pathway. The pathways in apoptosis then converge to activate caspases that cleave cellular proteins and break down cells.

D. Conclusion

1. The n-hexane, chloroform, and ethyl acetate extracts of *P. ostreatus* contain bioactive compounds of alkaloid, flavonoid, and terpenoid groups.
2. The *P. ostreatus* extracts that showed the best results in the cytotoxic test were ethyl acetate extracts against T47D cells with an IC50 value of 3.226 µg/mL and chloroform extracts against MCF7 cells with an IC50 value of 371.15 µg/mL.

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