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Phytochemical Analysis, Antioxidant Activity, and Cytotoxic Activity of Lentinula Edodes (Shiitake Mushroom) Extracts towards Breast Cancer Cell Line T47D

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Abstract: ***Breast cancer is a major threat as it is the number one cancer affecting women. Factors such as genetics, environment, and lifestyle play a role to cause cancer. Humans are constantly exposed to free radicals. An imbalance between free radicals and antioxidants will cause uncontrollable and excessive free radicals that cause cell damage which leads to uncontrollable cell growth. Shiitake mushroom (Lentinula edodes) is one of fungi that has been studied extensively on its health benefit as an antioxidant and anticancer. The study aims to investigate phytochemical constituent, antioxidant activity, and cytotoxicity of Lentinula edodes towards T47D breast cancer cells. Dry powder L. edodes was extracted with n-hexane, ethylacetate, and ethanol sequentially producing L. edodes extracts. Each extract is evaluated for its phytochemical constituents, antioxidant activity by DPPH assay, and cytotoxic activity using MTT assay towards T47D breast cancer cells. Phytochemical screening of L. edodes extracts showed it contains alkaloid, glycoside, flavonoid, and triterpenoid. DPPH assay of L. edodes ethanol extract resulted in active antioxidant activity on DPPH. Based on MTT assay, three L. edodes extracts resulted in very active cytotoxicity towards T47D cells.It ought to be pursued to develop L. edodes as an antioxidant and breast cancer therapeutic.***

Keywords: *Lentinula edodes,* shiitake mushroom, antioxidant, cytotoxicity, T47D cells

1. Introduction

Cancer is categorized into non-communicable disease which in recent years has become the leading cause of death world-wide.[1] It is not yet postulated on how cancer has an increasing incidence in the world; however it is suggested that the shifting socio-economic factors may play a role.[2,3] Worldwide, breast cancer is placed on the second rank on the most common diagnosed cancer in both sexes. In females, breast cancer is the most common diagnosed cancer and the leading cause of cancer death. According data from The Global Cancer Observatory done in 2018 and published in World Health Organization (WHO), breast cancer accounts for the majority cancer in Indonesian female.[1]

Predisposing factors such as age, disease history, breast pathology, family history, and genetics play a role in the development of breast cancer.[4]Breast cancer treatment is dependent on the stage and molecular profile of the disease. The treatments include breast-conserving therapy, mastectomy, postmastectomy radiation, neoadjuvant chemotherapy, chemotherapy, and endocrine therapy.[5] Doxorubicin, an anthracycline commonly used for breast cancer chemotherapy, has been known for its efficacy in the treatment of breast cancer. However, it also has been known for its devastating side effects if used in a long-term. It can cause severe liver damage, severe cardiac toxicity, and irreversible cardiomyopathy, therefore limiting the clinical use of doxorubicin.[6,7] Herbal medicines have been perceived as an alternative that offers less side effects, especially in Indonesian population that have a low to middle income. Additionally, it may cost less. Based on Andriati (2016), herbal medicines may offer better compliance.[8] This research will look further on Indonesian-cultivated *Lentinula edodes'* capability as an anticancer by investigating in its phytochemistry, antioxidant activity, and cytotoxicity toward T47D breast cancer cell line.

2. Literature Survey

Many mushroom species are now being explored into its antioxidant and anticancer activity. One of which is shiitake mushroom (*Lentinula edodes),* that can be easily found and grown in many countries. There are a handful of studies from different regions of the world studying the properties of *Lentinula edodes* extract as an antioxidant and anticancer. A study in Brazil conducted by Finimundy et al. shows a promising outcome in which *Lentinula edodes* has anti-proliferative effect on human tumor cell lines laryngeal carcinoma (HEp2) and cervical adenocarcinoma (HeLa).[9] Moreover, a study conducted in Indonesia by Ekowati has shown anticancer activity of *Lentinula edodes* against HeLa cells through investigating cytotoxicity and macrophages apoptotic and phagocytic activities.[10]

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3. Material and Methods

Material:
A dried Indonesian-cultivated *L. edodes* was bought from local supermarket and T47D breast cancer cell line was obtained from *Laboratoria Pengembangan Teknologi Industri Agro dan Biomedika, Badan Pengkajian dan Penerapan Bioteknologii* and cultured in Department of Medical Chemistry, Universtias Indonesia.

Maceration and Extraction of *Lentinula edodes*:
The dried *L. edodes* was grinded until it became powder consistency. Then, the sample is macerated in multilevel manner with three different solvents which have different polarity. The solvents are n-hexane, ethylacetate, ethanol, respectively. In order to facilitate the filtering process of the solution, filter paper is used to put away possible debris of the sample. For extraction, the filtrates are evaporated to obtain N-hexane, ethyl acetate, and ethanol extract of *L. edodes.*

Phytochemical Analysis:
To analyze the phytochemical constituents of *L. edodes,* we did thin layer chromatography (TLC) and phytochemical screening for saponin, flavonoid, tannin, glycoside, alkaloid, and triterpenoid/steroid.

Saponin
Extracts were diluted with 10mL of hot water which were then diluted. Two milliliters of extract were put into different reaction tubes. Then, the extracts were mixed by shaking the reaction tube. Let the extracts rest for ten minutes as it formed foam. Next, a drop of HCL 2N was added. Saponin was detected if the foam did not disappear.

Flavonoid
Extracts were diluted with 10mL of hot water which were then diluted. Two milliliters of extract were put into different reaction tubes. Then, 0.5mL of concentrated HCl was added to the tube followed by adding 4cm of Mg strip. Flavonoid was detected if red / orange / green color was observed.

Tannin
Extracts were diluted with 10mL of hot water which were then diluted. One milliliter of extract was put into different reaction tubes. Then, 1mL of 10% $FeCl_3$ was added. Tannin was detected if the extracts appeared as dark blue green.

Glycoside
Extracts were diluted with 10mL of hot water which were then diluted. One milliliter of extract was put into different reaction tubes. Then, the reaction tubes were heated. Next, 1 mL of ethyl acetate anhydride and 2 mL of concentrated H_2SO_4 were added. Glycoside was detected if the extracts appeared blue green.

Alkaloid
Each extract was added with 5mL of $CHCl_3$ and 2 drops of NH_4OH. Then, the extracts were filtered and vaporized. Next, 4mL of HCl 2N were added into each extract. Each extract was separated into three reaction tubes. The first tube was used as a blank that contained 1mL of the mixture that had been added with 2mL of HCL 2N. The second tube that contained 2mL of the mixture was added with 3 drops of Dragendorff, while the third tube that also contained 2mL of the mixture was added with 3 drops of Mayer. Alkaloid was detected if the second tube had orang precipitate and the third tube had yellow precipitate.

Triterpenoid or Steroid
Each sample (2mL) was added with 5 mL heated ethanol. Two milliliters of the mixture were put into a reaction tube which then was heated. Then, 0.5mL of $CHCl_3$, 0.5 mL of glacial acetic acetate, and 2mL of concentrated H_2SO_4 were added slowly by the wall of the reaction tube. Triterpenoid was detected if chocolate-violet ring was formed, whilst steroid was detected if the mixture appeared blue green.

Thin Layer Chromatography (TLC)
Thin layer chromatography used a TLC plate covered with a silica that acted as stationary phase. It had a length of 5 cm and an undetermined width – as long as it had sufficient space to place all three extracts on it. Two lines were drawn in top and bottom ends with a distance of 0.5 cm to the edge of TLC plate. On the bottom line, three dots were drawn to mark the place for each extract. After placing the extract, a chamber containing eluent solvents was prepared. The eluent solvents were N-hexane and ethyl acetate with a ratio of 7:1 which acted as a mobile phase. Then, the TLC plate was placed in the chamber until the mobile phase, eluent solvent, reached the top line. Finally, the TLC plate was placed under UV lamp to observe the spots that reflect the chemical compounds. Retention factor (R_f) was calculated through dividing the extract travel distance by the solvent travel distance.

DPPH Assay
Ethanol and ethyl acetate extracts of *L. edodes* were used to evaluate the antioxidant activity of the sample, whilst ascorbic acid was used as positive control to evaluate and compare the DPPH assay results. Ethanol extract, ethyl acetate extract, and ascorbic acid were made into five sequential concentrations from the highest to lowest by diluting them with ethanol. Ethanol extract's concentrations are 4,000; 2,000; 1,000; 500 and 250µg/mL, whilst ethylacetate extract's concentrations are 1,000, 500, 250, 125, and 62.5µg/mL. Ascorbic acid concentrations are 50, 25, 12.5, 6.25, and 3.75µg/mL. The DPPH reagent used had a concentration of 100ppm. The concentration was achieved through dilution with ethanol. The DPPH reagent was added to each reaction tube. The mixture was incubated for 30 minutes in a dark room. The result of the DPPH assay was evaluated through spectrophotometer with a wavelength of 517nm. The decreasing absorbance of the mixture indicated DPPH inhibition by the extract. The percentage of inhibition was calculated using the formula below.

$$Inhibition\ (\%) = \frac{Abs\ Control - Abs\ solution\ tested}{Abs\ Control} x\ 100$$

The inhibition percentages were plotted against the logarithmic concentration of the extract to produce linear regression equation. From the linear regression equation, the IC50 values for each extract were calculated.

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MTT Assay
Cultured cancer lines were counted in the improved Neubauer counting chamber. Then, it was prepared and transferred into a 96-wells plate for 24 hours inoculation. Each well was filled with different concentrations of extract, negative, and positive control. Each extract and positive control had 6 varying concentrations which are 200; 10; 50; 25; 12.5; 6.25; 3.125and 1.5625µg/mL. DMSO was used as negative control, while doxorubicin was used as the positive control of the extract. Then, it was incubated for another 24 hours. Afterwards, a dissolved MTT reagent in DPBS was placed into each well. Then, it was incubated for 4 hours. Finally, the assay was evaluated through Visible Spectrophotometer with a wavelength of 630nm. A higher absorbance indicated a higher concentration of viable cells. The percentage of inhibition was calculated using the formula below.

$$\text{Inhibtion } (\%) = \frac{Abs\ Control - Abs\ solution\ tested}{Abs\ Control} x100\ \%$$

The inhibition percentages were plotted against the logarithmic concentration of the extract to produce linear regression equation. From the linear regression equation, the IC50 values for each extract were calculated.

4. Results

Phytochemical Analysis
Lentinula edodes' extracts are labelled based on its solvent. To simplify, extract in n-hexane is labelled as LHE, extract in ethanol is LEE, and extract in ethyl acetate is LAE. Screened constituents include saponin, flavonoid, tannin, glycoside, triterpenoid, steroid, and alkaloid. Phytochemical analysis' results in Table 1 show that all three extracts consist of triterpenoid. Both LHE and LEE consist of glycoside and alkaloid, while LAE consists of flavonoid. As seen on the Figure 1 of *L. edodes'* TLC chromatogram,each spot represents the travel distance of the mobile phase. The travel distance calculated from the bottom edge for both the mobile phase and solvent. Rf value is measured through dividing the travel distance of the mobile to the travel distance of the solvent. The Rf value in Table 2 reflects the spots as seen on Figure 1. They are written in sequence based on the spots in TLC, starting from the bottom edge to the top edge.

Table 1: Phytochemical Screening

	Saponin	Flavonoid	Tannin	Glycoside	Alkaloid	Triterpenoid/ Steroid
LHE	-	-	-	+	+	Triterpenoid
LEE	-	-	-	+	+	Triterpenoid
LAE	-	+	-	-	-	Triterpenoid

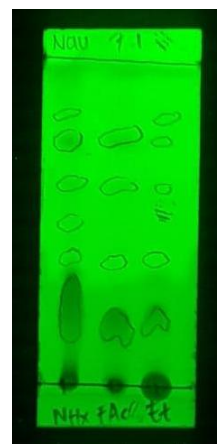

Figure 1: Thin Layer Chromatography (TLC) Chromatogram of *L. Edodes* extracts
N-Hx:n-Hexane Extract; **EAc:** Ethylacetate Extract; **Et:** Ethanol extract

Table 2: Thin Layer Chromatography

	LHE	LEE	LAE
Rf Value	0.225; 0.4; 0.5; 0.625; 0.75; 0.95	0.25; 0.275; 0.725; 0.75; 0.95	0.175; 0.375; 0.6; 0.75
Phytochemical Components	6	5	4

Antioxidant Activity of *L. edodes* extract on DPPH Assay
The following results of DPPH assay of ethylacetate and ethanol extract of *L.edodes*, exclude n-hexane extract because it is a non-polar extract which not able to dissolve well in ethanol solvent, but include ascorbic acid as a positive control. The assay was conducted with three replications. The IC50 value for LEE, LAE, and ascorbic acid obtained are 16.6; 1,282.37; and 0.69 µg/mL respectively (see Table 3). The IC50 values for LEE and LAE were calculated using the linear regression equation obtained from plotting the percentage inhibition to the logarithmic concentration of the extracts (see Figure 2 and Figure 3).

Table 3. Antioxidant activity (IC50) of *L.edodes* extracts Ascorbic acid on DPPH

Extract	IC50 (µg/mL)
LEE	16,6
LAE	1.282,37
Ascorbic acid	0.69

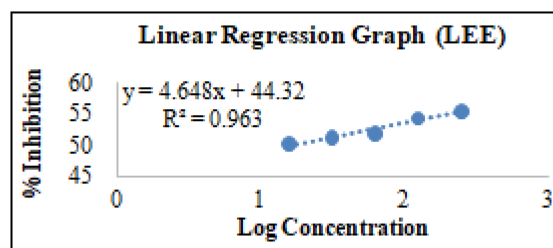

Figure 2: LEE Linear Regression on DPPH

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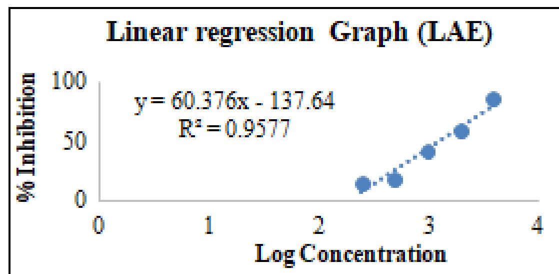


Figure 3: LAE Linear Regression on DPPH

Cytotoxic Activity of *L. edodes* extracts against T47D Cells

Table 4 describes the result of *L. edodes* extracts and doxorubicin, as positive control, by MTT assay towards T47D breast cancer cell line. Linear regression of LEE, LHE and LAE on T47D cancer cells are displayed in Figure 4-6. IC50 values of all three extracts are low, <20 µg/mL. LHE has the lowest IC50 which is 1.4561E-11 µg/mL, followed by LAE and LEE which are 0.000769691 µg/mL and 9.52472058 µg/mL respectively. In this research, doxorubicin is used as positive control. It resulted in 6.58119E-23 µg/mL.

Table 4: Cytotoxicity of *L. edodes* extracts and doxorubicin against T47D Cells

	LHE	LEE	LAE	Doxorubicin
Trial 1 (µg/mL)	3.61851E-17	0.006517959	0.001539219	1.31624E-22
Trial 2 (µg/mL)	2.9122E-11	19.04292322	9.87094E-12	6.58809E-29
Mean (µg/mL)	1.4561E-11	9.52472059	0.000769691	6.58119E-23

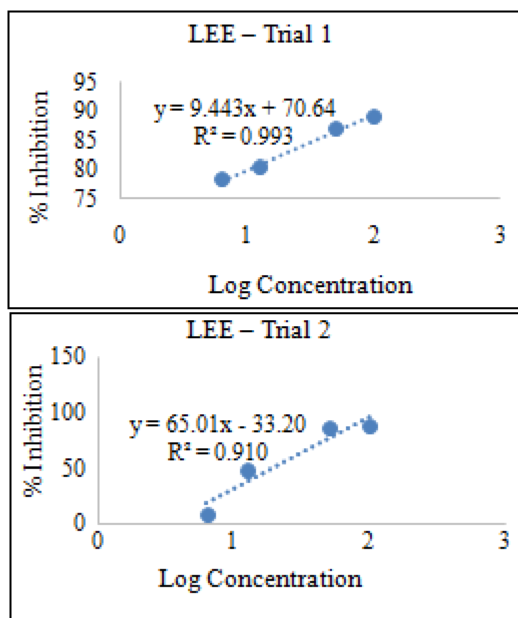
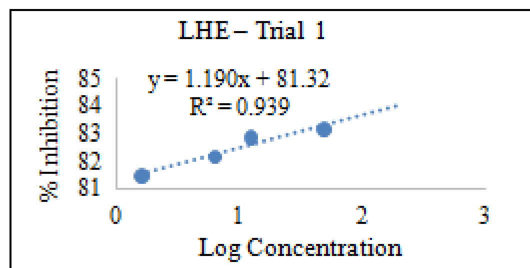


Figure 4: LEE Linear Regression on T47D cells



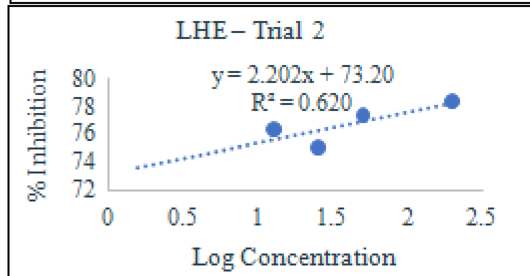
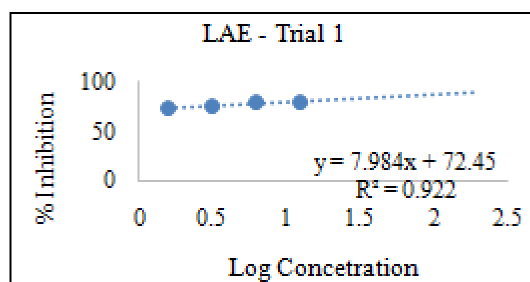


Figure 5: LHE Linear Regression on T47D cells



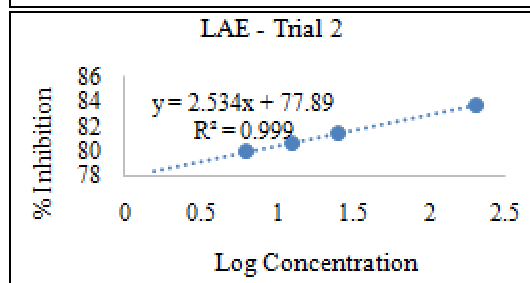


Figure 6: LAE Linear Regression on T47D cells

5. Discussion

Phytochemical Analysis of *Lentinula edodes*

Secondary metabolites of *Lentinula edodes* were examined through thin layer chromatography and phytochemical analysis. These tests are used to identify the phytochemical constituents in categorical manner. In this research, the phytochemical compounds that are being examined are saponin, flavonoid, tannin, glycoside, triterpenoid, steroid, and alkaloid. These compounds are naturally found in plants. It exhibits physiological functions and benefits when administered as drugs, though mechanisms of it are mostly not understood yet.[11]Based on Choi et al (2016), they have found Korean-cultivated *L. edodes* ethanolic extract to have phytochemical constituent of polyphenols, flavonoid, β-carotene, and lycopene.[12] Therefore, further screening of the phytochemical constituents should be explored. However, different cultivation area may result in different phytochemical constituent.[13] The secondary metabolites found in the preliminary screening are responsible for the antioxidant cytotoxic activity exhibited by the *L. edodes* extracts. The secondary metabolites found in the preliminary

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screening are responsible for the antioxidant cytotoxic activity exhibited by the *L. edodes* extracts. Flavonoid contributes to antioxidant activity.[14], whilst glycosides[15], alkaloids[16], and triterpenoids[17-18] contribute tocytotoxic activity towards the cancer line.TLC analysis of all three extracts of *L. edodes* showed they contained twelve phytochemical constituents. The extract with the majority of phytochemical constituents was LHE, with 6 constituents. All three extracts had one constituent in common Rf value of 0.75, however only LHE and LEE have in common 0.95 as their Rf value.

DPPH Assay of *Lentinula edodes*

DPPH assay contributes in evaluating scavenging activity of the fungal extract. DPPH acts as a stable free radical that can accept either electrons or hydrogen. Adding antioxidative fungal extract will then reduce and stabilize DPPH. The absorbance of reduced DPPH will be then read in visible spectrophotometer at the wavelength of 517nm to evaluate the scavenging activity of the extract.[19-20]DPPH with no extract is used as negative control, while ascorbic acid is used as positive control. In comparison, LEE with IC50 of 16.6 µg/mL has a lower value than LAE with IC50 value of 1,282.37 µg/mL. Therefore, LEE has a greater scavenging activity than LAE because IC50 is defined as the minimum extract concentration to scavenge ROS to 50%[21], in this case free radical DPPH. Marjoni et al.25[22] describes classification of antioxidant activity based on the IC50. With that classification, LEE is considered as a very active antioxidant because it has IC50 value less 50 µg/mL, while LAE is considered as not active because it has IC50 value greater than 500 µg/mL. To compare with ascorbic acid the two extracts have a higher IC50 value. Therefore, the antioxidant activity of the extracts is lower than the ascorbic acid. Furthermore, Cheung LM et al.[23] evaluated Hong Kong-cultivated *L. edodes*ethanol extractfor its scavenging activity to inhibit lipid peroxidation in rats by determining its IC50 value. The extract was evaluated for its total phenolic compound and its IC50 value of antioxidant activity. It was found out that the IC50 value and total phenolic compound have a strong negative correlation.

MTT Assay of *Lentinula edodes*

LHE, LEE, and LAE extracts of *L. edodes* showed a cytotoxic activity and considered to be very active towards the T47D breast cancer cell line as per definition by American Cancer Institute and Atjanasuppat et al (2009), respectively. The cytotoxic ability of the extract is represented by its IC50 value, which is defined as the concentration of the tested substance that can inhibit 50% of cancer cell growth. According to American Cancer Institute, herbal extract is considered to have cytotoxic activity if they present with IC50 value below <20µg/ml.[24] Additionally, the result of the MTT assay of *L. edodes* are considered to be very active (<20µg/ml) as per definition of Atjanasuppat et al (2009).[25] To compare with the doxorubicin, all the extracts had a higher IC50 values. This indicates doxorubicin as positive control is more effective than the extracts. More repetition of the MTT assay should be repeated to have more accurate results. Israilides et al (2008) investigated the cytotoxic effect of *L. edodes* towards MCF-7 breast cancer and normal cells by MTT assay.[26]*L. edodes* has a low IC50 value to inhibit MCF-7 breast cancer cell proliferation. The IC50 was 73±14µg/ml.[13]

6. Conclusion

TLC analysis was able to identify that there are 12 phytochemical constituents of *L. edodes*. Whereas there are4 phytochemical constituents that are identified with preliminary phytochemical screening. These include flavonoid, glycoside, alkaloid, and triterpenoid. The DPPH assay concluded that the ethanol extract of *Lentinula edodes* is very active as an antioxidant on DPPH, whilst ethylacetate extract is not active. The MTT assay of *L. edodes* extracts resulted in a very active cytotoxic activity towards the T47D breast cancer cell line.

7. Future Scope

Lentinula edodes extracts demonstrated an active antioxidant and strong cytotoxicity against T47D cells. Thus, it is important to continue investigating and developing Lentinula edodes extracts as a natural antioxidant and anticancer agent for breast cancer treatment.

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Conflict of Interest

The author states no conflict of interest.

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