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Focus and Scope

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The Cytotoxic Effect of Ganoderma lucidum Ethanol Extract on KB CCL-17 Oral Cancer Cell Culture

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Abstract

Abstrak

Kanker rongga mulut adalah neoplasma ganas yang menyerang rongga mulut dan penyebab kematian tertinggi.
Ganoderma lucidum (G. lucidum) merupakan bahan alam yang memiliki efek antikanker. Sel kanker KB CCL-17
adalah model kanker yang berasal dari epidermal karsinoma mulut. Tujuan penelitian ini adalah mengetahui
pengaruh aktivitas sitotoksik setelah pemberian ekstrak etanol jamur G. lucidum dan Cisplatin pada kultur sel
kanker rongga mulut KB CCL-17. Evaluasi sitotoksik dengan Methyl Thiazolyl Tetrazolium (MTT) assay pada
kultur sel rongga mulut KB CCL-17 yang dibagi menjadi 20 kelompok yaitu 10 kelompok kontrol (M: media, K:
tidak diberi perlakuan, C: diberi Cisplatin dengan konsentrasi 250; 150; 62,5; 31,25; 15,6; 7,8; 3,9; dan 1,95
µg/mL), 10 kelompok perlakuan (KP: diberi ekstrak etanol G. lucidum konsentrasi 1.000; 500; 250; 125; 62,5;
31,25; 15,6; 7,8; 3,9; dan 1,95 µg/mL). Aktivitas sitotoksik diukur berdasarkan nilai inhibitory concentration 50%
(IC50). Ekstrak etanol jamur G. lucidum memiliki aktivitas sitotoksik sel kanker rongga mulut KB CCL-17 yaitu
nilai IC50 sebesar 8,49 µg/mL. IC50 Cisplatin terhadap sel kanker rongga mulut KB CCL-17 sebesar 11,55 µg/mL.
Terdapat pengaruh aktivitas sitotoksik setelah pemberian ekstrak etanol G. lucidum dan pemberian Cisplatin pada
kultur sel kanker rongga mulut KB CCL-17.

Kata kunci: Ganoderma lucidum, IC50, kanker rongga mulut, sel KB CCL-17, sitotoksik

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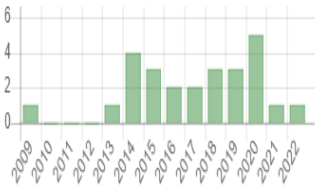

The Cytotoxic Effect of Ganoderma lucidum Ethanol Extract on KB CCL-17 Oral Cancer Cell Culture

Aktivitas Sitotoksik Ekstrak Etanol Jamur Ganoderma lucidum pada Kultur Sel Kanker Rongga Mulut KB CCL-17

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Abstrak

Kanker rongga mulut adalah neoplasma ganas yang menyerang rongga mulut dan penyebab kematian tertinggi. Ganoderma lucidum *(*G. lucidum*) merupakan bahan alam yang memiliki efek antikanker. Sel kanker KB CCL-17 adalah model kanker yang berasal dari epidermal karsinoma mulut. Tujuan penelitian ini adalah mengetahui pengaruh aktivitas sitotoksik setelah pemberian ekstrak etanol jamur* G. lucidum *dan Cisplatin pada kultur sel kanker rongga mulut KB CCL-17. Evaluasi sitotoksik dengan* Methyl Thiazolyl Tetrazolium *(MTT) assay pada kultur sel rongga mulut KB CCL-17 yang dibagi menjadi 20 kelompok yaitu 10 kelompok kontrol (M: media, K: tidak diberi perlakuan, C: diberi Cisplatin dengan konsentrasi 250; 150; 62,5; 31,25; 15,6; 7,8; 3,9; dan 1,95 µg/mL), 10 kelompok perlakuan (KP: diberi ekstrak etanol* G. lucidum *konsentrasi 1.000; 500; 250; 125; 62,5; 31,25; 15,6; 7,8; 3,9; dan 1,95 µg/mL). Aktivitas sitotoksik diukur berdasarkan nilai inhibitory concentration 50% (IC_{50}). Ekstrak etanol jamur* G. lucidum *memiliki aktivitas sitotoksik sel kanker rongga mulut KB CCL-17 yaitu nilai IC_{50} sebesar 8,49 µg/mL. IC_{50} Cisplatin terhadap sel kanker rongga mulut KB CCL-17 sebesar 11,55 µg/mL. Terdapat pengaruh aktivitas sitotoksik setelah pemberian ekstrak etanol* G. lucidum *dan pemberian Cisplatin pada kultur sel kanker rongga mulut KB CCL-17.*

***Kata kunci**:* Ganoderma lucidum, *IC_{50}, kanker rongga mulut, sel KB CCL-17, sitotoksik*

Abstract

Oral cancer is a malignant neoplasm that attacks the oral cavity and has the highest cause of death. *Ganoderma lucidum* (*G. lucidum*) is a natural material that has anticancer effects. KB CCL-17 cancer cell is a model of cancer originating from epidermal oral carcinoma. The aim of this study to determine the cytotoxic effect after administration of ethanol extract of *G. lucidum* and Cisplatin on KB CCL-17 oral cancer cell culture. *G. lucidum* ethanol extract was obtained by maceration method using 96% ethanol solvent. Cytotoxic evaluation with methyl thiazolyl tetrazolium (MTT) assay was performed on KB CCL-17 oral mucous cell cultures divided into 20 groups. Ten control groups (M: media, K: not treated, C: given Cisplatin concentrations of 250;150;62.5;31.25; 15.6;7.8;3.9;1.95 µg/mL), 10 treatment groups (KP: given ethanol extract *G. lucidum* concentrations of 1,000; 500;250;125;62.5;31.25;15.6;7.8;3.9;1.95 µg/mL). The cytotoxic effect was measured based on inhibitory concentration 50% (IC_{50}) values. The IC_{50} of ethanol extract of *G. lucidum* on KB CCL-17 oral cancer cell is 8.49 µg/mL and IC_{50} Cisplatin against oral cancer cells KB CCL-17 is 11.55 µg/mL. There is an effect of cytotoxic activity of the ethanol extract of *G. lucidum* and Cisplatin in oral cancer cell cultures of KB CCL-17.

Keywords: cytotoxic, *Ganoderma lucidum*, IC_{50}, KB CCL-17 cells, oral cancer

Introduction

Cancer is a disease that has a high mortality rate in the world. There were 14.1 million new cancer cases and caused 8.2 million deaths worldwide in 2012[1]. Oral cancer is one of the cancers and occupies the sixth position of eight cancers that often occur. Oral squamous cell carcinoma is a common oral cancer that is around 90% of oral cancer. Risk factors for this disease are smoking and alcohol consumption[2].

The utilization of natural ingredients used as medicines rarely causes adverse side effects compared to drugs made from synthetic materials. Many research is done by utilizing natural ingredients which all aim to produce medicines to support health care programs. One of the natural ingredients that have anticancer effects is *Ganoderma lucidum* (*G. lucidum*)[3].

G. lucidum has functioned as an antibacterial, antioxidant, anticancer, decrease blood pressure, decrease blood cholesterol, an inhibitor of platelet collection, and immunomodulatory protein[4]. *G. lucidum* also has the potential as an anticancer in HeLa cervical cancer cells[5]. *G. lucidum* contains several bioactive compounds that can be used as anticancer drugs. These compounds include triterpenoids and polysaccharides[3].

Triterpenoids induce apoptosis in human cancer cells through intrinsic pathways followed by caspase cascade activation[6]. The intrinsic pathway is an apoptotic pathway that begins with the release of cytochrome-c from mitochondria to the plasma membrane cells, the release of enzymes depends on the membrane permeability regulated by the *B Cell Lymphoma-2* (BCL-2) protein. Triterpenoids function to increase the activity of p53 and proapoptotic proteins such as *BCL-2 Associated X* (BAX), decrease antiapoptotic proteins such as BCL-2, and induce *G1 Cell Cycle Arrest* by inhibiting beta catenin[3,7].

Polysaccharides have been shown to increase the host immune response by stimulating the production of macrophages, *Natural Killer* (NK) cells, and T-lymphocytes. Polysaccharides can act as antioxidants by reducing oxidative damage caused by *Reactive Oxygen Species* (ROS) and preventing the breaking of the *Deoxyribonucleic Acid* (DNA) strand. Polysaccharides also prevent tumor-derived angiogenesis by reducing the proliferation of *Human Umbilical Vein Endothelial Cells* (HUVEC) and inhibiting the secretion of angiogenic factors such as *Vascular Endothelial Growth Factor* (VEGF) and *Transforming Growth Factor Beta 1* (TGF-β1)[3]. The active compound polysaccharide can inhibit cell proliferation and induce apoptosis through the *caspase-dependent pathway* by increasing the protein Fas, caspase-8, and caspase-3 in colon cancer cells[8].

Based on the description above, there were no studies related to the cytotoxic effect of *G. lucidum* ethanol extract on oral cancer cell culture. So, the aim of this study to determine the cytotoxic effect after administration of ethanol extract of *G. lucidum* and Cisplatin on KB CCL-17 oral cancer cell culture.

Methods

This study has received ethical approval from the Health Research Ethics Commission of the Faculty of Medicine, Jenderal Soedirman University Number: 272/KEPK/X/2018. This type of research is an experimental research design with a *post-test only control group design*.

The samples of this study are KB CCL-17 oral cancer cells which are oral epithelial cancer cells cultured with *Dulbecco's Modified Eagle Medium* (DMEM) growth media. The number of samples was selected by a *simple random sampling technique*. The ethanol extract of the *G. lucidum* will be tested for its cytotoxicity effect through a cytotoxicity test using the enzymatic reaction *Methyl Thiazolyl Tetrazolium* (MTT) which is expressed as an inhibitory concentration 50% (IC_{50}) value.

a. Manufacture of *G. lucidum* extract.

The *G. lucidum* fungi were obtained from a farmer in Salahuni village, Cianjur. The fungus was determined in the Mycology Laboratory of the Faculty of Biology, Jenderal Soedirman University. The method used in the extraction of this fungus is maceration with 96% ethanol solvent. The fungus *G. lucidum* is made in dry powder, then 100 grams of dry powder is put into the macerator and added with 1,000 mL of 96% ethanol. Material soaked 3x24 hours. The material is filtered with filter paper and put into a *rotary evaporator* with a speed of 280 rpm and a temperature of 60 ^{o}C for three hours[9]. The results of high viscosity extracts of *G. lucidum* were 6.82 g.

b. Activation of oral cancer cell cultures

Activation of oral cancer cell cultures (KB CCL-17) was conducted at the Integrated Research and Testing Laboratory (Laboratorium Penelitian dan Pengujian Terpadu - LPPT) Unit 3 of Gadjah Mada University, Yogyakarta. KB CCL-17 cells were taken from a liquid nitrogen tank and resolved in a 37°C water bath. The cells are poured into centrifugation tubes containing 10 mL DMEM, 10% fetal bovine serum (FBS), 3% penicillin-streptomycin, and 1% *fungizone* in the *laminary airflow chamber*. Cells were centrifuged for 10 minutes at 1200 rpm. The supernatant is removed and the precipitate formed is added with DMEM-serum and then allowed to stand for 20 minutes, then centrifuged at a speed of 1200 rpm for 10 minutes. Cell suspensions were added to a *tissue culture flask* (TCF) with growth media containing 20% FBS. TCF was incubated in an incubator at 37°C and 5% CO_2 with loosened the cap[10,11].

c. Preparation of test compound solutions

Ethanol extract of *G. lucidum* 2 mg with 1 mL of DMEM, to obtain a solution with a concentration of 2,000 µg/mL. The test solution was then diluted 2 times to obtain a concentration of 1,000 µg/mL which was then used in serial dilutions for the treatment group. The control solution is made by dissolving Cisplatin (the gold standard for cancer treatment) which has been available in the form of a 1 mg/mL concentration of Kalbe© production. The same serial dilution was carried out for the control group[11].

d. KB CCL-17 cell culture

Cells were centrifuged for five minutes at a speed of 2,000 rpm, the supernatant was removed and 1 mL of suspension was left for pellet resuspension. DMEM with 10% FBS content is added to the cell suspension. Cells are distributed on several TCFs and new media is replaced. Furthermore, cells were stored in an incubator at 37^{o}C and 5% CO_2[10,11].

e. Harvesting KB CCL-17 cells

Cells were taken from CO_2 incubator and evaluated using an *inverted* microscope. After 80% confluent, the media was removed with a sterile Pasteur pipette and washed twice with PBS, 50 µL trypsin-EDTA 0.24% was added to the cell. Inactivation of trypsin is done by adding 2-3 mL of serum DMEM. Cells were incubated in an incubator at 37°C for 5 minutes interrupted centrifuged with 1200 rpm rotation for 10 minutes at room temperature[10,11].

f. Cytotoxicity test

Evaluation of cytotoxic activity was carried out using a *96 well microplate* and 2x10^4 cells/*well*. The ethanol extract of *G. lucidum* was added to certain *wells* with concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, and 1.95 µg/mL[12]. Cisplatin as a positive control was added to *wells* with concentrations of 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, and 1.95 µg/mL[13]. Then cells were incubated for 24 hours. MTT reagent concentration of 5 mg/mL and *stopper* put in each *well* then placed in a dark room at room temperature for 24 hours. The sample is inserted into the

enzyme-linked immunosorbent assay (*ELISA) Reader* with a wavelength of 550 nm[14]. Decreased absorbance values indicate decreased cell proliferation. IC_{50} values are calculated using Probit Tables.

Result

G. lucidum powder (100 g) was used as a raw material in the extract production using the maceration method. The result of *G. lucidum* extract is 6.82 grams. The MTT assay method was used in the cytotoxic test of *G. lucidum* ethanol extract against cancer cells in the oral cavity KB CCL-17. The results are showing the absorbance value at 96 *well-plates*. The absorbance results show the number of living cells capable of reacting to reagents and creating color changes. Table 1 shows the absorbance values of the media control group, cell control, Cisplatin, and ethanol extract of *G. lucidum* obtained from *ELISA Reader*.

There was a decrease in absorbance value with increasing concentration, both in the ethanol extract group *G. lucidum* and the Cisplatin group. The absorbance value in each group is then used to calculate the percentage of living cells. Oral cancer cell death is obtained by 100 % reduced percent of living cells.

The percentage of death cell is then used to calculate the IC_{50} value. IC_{50} is a parameter of compounds with cytostatic properties inhibiting the growth of cancer cells by 50%. Test compounds have toxic properties after 24 hours of contact with a value of IC_{50} less than 1000 µg/mL. IC_{50} values were obtained by calculating the logarithm of the concentration of ethanol extract of *G. lucidum* and Cisplatin and changing the percentage of cell death into Probit Table (Table 2).

The relation between independent variable or x (extract ethanol mushroom *G. lucidum* and Cisplatin) against the activity of cytotoxic (y) on oral cell cancer is calculated using the equation regression linear. Based on the data in Table 2, the line equation is obtained, namely y=0.46x + 487 for ethanol extract of *G. lucidum* and y=1,328x + 3,587 for Cisplatin.

IC_{50} value of ethanol extract of *G. lucidum* was 8.49 µg/mL and Cisplatin was IC_{50} = 11.55 µg/mL.

Table 1. Absorbance values after administration of ethanol extract of *G. lucidum* and Cisplatin

No.	Research group	Average Absorbance
1	Media control	0.034
2	Cell control	0.819
3	*G. lucidum* 1.95 µg/mL	0.393
4	*G. lucidum* 3.9 µg/mL	0.348
5	*G. lucidum* 7.8 µg/mL	0.315
6	*G. lucidum* 15.62 µg/mL	0.306
7	*G. lucidum* 31.35 µg/mL	0.271
8	*G. lucidum* 62.5 µg/mL	0.243
9	*G. lucidum* 125 µg/mL	0.192
10	*G. lucidum* 250 µg/mL	0.115
11	*G. lucidum* 500 µg/mL	0.065
12	*G. lucidum* 1,000 µg/mL	0.030
13	Cisplatin 1.56 µg/mL	1.157
14	Cisplatin 3.125 µg/mL	1,038
15	Cisplatin 6.25 µg/mL	0.964
16	Cisplatin 12.5 µg/mL	0.871
17	Cisplatin 25 µg/mL	0.198
18	Cisplatin 50 µg/mL	0.072
19	Cisplatin 100 µg/mL	0.062
20	Cisplatin 200 µg/mL	0.071

Table 2. Results of Probit Analysis of Cytotoxic Test of *G. lucidum* Ethanol Extract

No	Group	Concentration Log (X)	CCL-17 KB cells	
			Cell death (%)	Probit (Y)
1	*G. lucidum* 1.95 µg/mL	0.29	53.94%	5.1
2	*G. lucidum* 3.9 µg/mL	0.59	59.21%	5.23
3	*G. lucidum* 7.8 µg/mL	0.89	63.08%	5.33
4	*G. lucidum* 15.62 µg/mL	1.19	64.13%	5.36
5	*G. lucidum* 31.35 µg/mL	1.49	67.42%	5.44
6	*G. lucidum* 62.5 µg/mL	1.79	71.52%	5.58
7	*G. lucidum* 125 µg/mL	2.09	77.49%	5.74
8	*G. lucidum* 250 µg/mL	2.39	86.52%	6.13
9	*G. lucidum* 500 µg/mL	2.69	92.38%	6.34
10	*G. lucidum* 1,000 µg/mL	3	89.45%	6.23
11	Cisplatin 1.56 µg/mL	0.19	8.89%	3.66
12	Cisplatin 3.125 µg/mL	0.49	18.26%	4.08
13	Cisplatin 6.25 µg/mL	0.79	24.09%	4.29
14	Cisplatin 12.5 µg/mL	1.09	58.41%	5.2
15	Cisplatin 25 µg/mL	1.39	84.4%	5.99
16	Cisplatin 50 µg/mL	1.69	94.33%	6.55
17	Cisplatin 100 µg/mL	2	95.11%	6.64
18	Cisplatin 200 µg/mL	2.30	70.85%	5.55

Discussion

The results of cytotoxic tests in this study showed the morphology of oral cancer cell KB CCL-17 after treatment decreased living cell colonies compared to control cells that were not treated. The higher concentration of ethanol extract from *G. lucidum* and Cisplatin will decrease the absorbance value which depicts the metabolic activity of living cells is decreases[5]. A decrease in value of absorbance with increasing concentrations of *G. lucidum* ethanol extracts and Cisplatin shows an increase in amount of cells that die and decreasing in amount of live cells. The concentration of ethanol extract of *G. lucidum* and Cisplatin which was able to inhibit 50% of test cells was 8.49 µg/mL and 11.55 µg/mL.

G. lucidum extract has cytotoxic activity in cancer cells and has been proven to induce apoptosis. Values of IC_{50} extracts of *G. lucidum* on studies of other cancer cells, such as breast cancer cells SUM-149 (0.25 mg/mL), PC3 prostate cancer cells (5.38 ug/mL), and DU145 prostate cancer cells (16.92 µg/mL)[9]. IC_{50} value of *G. lucidum* extract varies due to differences in extraction methods, solvents, and cancer *cell lines* used[15]. In this study, the cytotoxic activity of *G. lucidum* ethanol extract against cancer cells in the oral cavity KB CCL-17 was characterized by an IC_{50} value is 8.49 µg/mL.

IC_{50} value of *G. lucidum* extract obtained in this study is still in the range of IC_{50} value of *G. lucidum* extract in previous studies. This is caused by the KB CCL-17 cell type as epidermoid carcinoma just like

lung, cervical, breast, vaginal, and prostate carcinoma. KB CCL-17 cells as one of the epidermoid cancers have the characteristics of fast growth, recurrence, and metastasis. The KB CCL-17 cell morphology is in the form of epithelial tissue and is known to produce keratin[16,17].

Several studies report that the compounds contained in the ethanol extract of *G. lucidum* that have cytotoxic activity include triterpenoids and polysaccharides[9]. Extraction in this study uses ethanol to maintain and increase triterpenoid production[18]. Triterpenoids can be extracted using organic solvents such as methanol, ethanol, chloroform, or ether followed by different separation methods. The extraction method using organic solvents has higher cytotoxic activity in cancer cells than the extraction method using water. Triterpenoids are the main active ingredients that are extracted from organic solvents, and polysaccharides are the main active ingredients that are extracted from water. This shows that triterpenoids have a stronger cytotoxic effect than polysaccharides.

Triterpenoids have been shown to induce inhibition of cell cycle at the G1 phase by lowering the Cyclin D1 regulatory pathway via modulation of β-catenin and the G2 phase to suppress the activity of protein kinase C. Triterpenoids also induce apoptosis in the cancer *cell line* via the mitochondrial pathway followed by *caspase cascade* activation. Triterpenoids are proven to prevent tumor metastases by regulating *matrix metalloproteinase* (MMP), interleukin-8 (IL-8), and suppressing the secretion of inflammatory cytokines in macrophage cells. Triterpenoids can increase p53 and BAX which result in mitochondrial dysfunction and release cytochrome c to the cytosol. Cytochrome c will activate *caspase-3* to trigger the process of apoptosis[3,5,15].

IC_{50} Cisplatin value in this study (11.55 μg/mL) was higher than IC_{50} ethanol extract of *G. lucidum*. Cisplatin is one of the most effective anticancer drugs widely used in the treatment of cancer including head and neck, lungs, ovaries, leukemia, breast, and testicular cancer. Cisplatin is considered a cytotoxic drug that kills cancer cells by damaging DNA, inhibiting DNA synthesis and mitosis, and inducing apoptotic cells. Several molecular mechanisms of oxidative stress induction Cisplatin are characterized by the production of Reactive Oxygen Species (ROS) and fat peroxide, induces p53 signal and protects the cell cycle, regulates proto-oncogene reduction, and anti-apoptotic protein, and activates the intrinsic and extrinsic apoptotic pathway[19]. IC_{50} Cisplatin value in other studies of A549 lung cancer cells was 30 μg/mL and H2170 was 7 μg/mL[20].

The ethanol extract of *G. lucidum* in this study was proven to have a cytotoxic effect against KB CCL-17 oral cancer cells as well as Cisplatin. The results of this study indicate that ethanol extract of *G. lucidum* has the potential to be developed as a chemopreventive agent against oral cancer.

Conclusion

There is a cytotoxic effect after administration of *G. lucidum* ethanol extract and Cisplatin on KB CCL-17 oral cancer cell culture

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