





# The Effect of Ethanol Extract of Lingzhi Mushroom (*Ganoderma lucidum*) on Caspase-3 Expression in Oral Cancer Cells

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**Keywords:** Lingzhi mushroom, *Ganoderma lucidum*, caspase, KB CCL-17 cells.


**Abstract:** Oral carcinoma is cancer found in the mouth, lips, palate, gingiva, mouth floor, and cheek mucosa. Oral carcinoma is a common cause of death in Indonesia. The development of cancer cells in the oral cavity is affected by the loss of caspase-3 expression. A treatment using lingzhi mushroom is known to increase caspase-3 expression in cancer. This study aimed to know about the effect of ethanol extract of *Ganoderma Lucidum* on caspase-3 expression in oral carcinoma KB CCL-17. The samples were oral carcinoma KB CCL-17 cells with five treated groups 8.49 µg/ml (P1), 4.24 µg/ml (P2), 2.2 µg/ml (P3), 11.55 µg/ml (cisplatin), and one control group (K). Caspase-3 expression was analyzed using the immunocytochemistry method by counting the percentage of caspase-3 expression cells. The data were statistically analyzed using One Way ANOVA and Post Hoc LSD. The results of caspase-3 expression on each groups were 5.21 % (P1), 2 % (P2), 0.96 % (P3), 9.67 % (cisplatin) and 0.41 % (K). Ethanol extract of *Ganoderma Lucidum* increased caspase-3 expression in KB CCL-17 cells along with the increase of the dosage. The dosage of 8.96 µg/ml showed a higher increase of caspase-3 expression than the dosages of 4.24 µg/ml and 2.12 µg/ml. An effect of ethanol extract of lingzhi mushroom on caspase-3 expression in oral carcinoma KB-CCL17. The study using lingzhi mushroom should be more developed to determine the anti-cancer effect through various pathways.


## 1 INTRODUCTION


Cancer is one of the deadly diseases that provides 17.2 million cases worldwide and 8.9 million deaths in 2016. Cancer cases increased by 28% between 2006 and 2016 (Global burden diseases, 2016). Indonesia has a cancer prevalence that attacks all ages of 4.1% or an estimated 347,792 people (Risked, 2013). The importance of oral cancer was underscored in a recent publication on the burden of cancer on member countries where oral cancer was the fifth most common cancer among ASEAN member countries contributing to 50% of all new cancer cases (Cheong et al, 2018). 95% of cancer cases in the oral cavity are oral squamous cell carcinomas which appear in the form of lumps, white or red ulcers that often attack the lips, lateral tongue, gum, palate, and floor of the mouth (Scully & Kirby,

2014). Cancer cells can develop because molecularly they have interference with cell death or apoptosis program. One of the proteins that regulate the course of apoptosis in cells, namely Caspase-3, these proteins have become a target in cancer therapy development. The management of oral squamous cell cancer generally consists of surgery, radiotherapy, chemotherapy, or a combination. However, the actions needed to overcome this malignancy have various shortcomings that can harm patients. Therefore alternative cancer cell treatments are currently being attempted through various studies. Many studies have been carried out using natural ingredients, all of that aim to produce medicines to support the health care program. Also, the use of natural ingredients used as medicine rarely causes adverse side effects than medicine made from synthetic materials. One of the natural materials that

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have an anti-cancer effect is the *Ganoderma Lucidum* fungus.

Research proved that the ethanol extract of *Ganoderma sp.* mycelium Banyumas 1 isolate can be an anti-cancer in HeLa cervical cancer cells. *Ganoderma sp.* contains several bioactive compounds that can be used as medicine with anti-cancer properties (Hidayati et al, 2014). These compounds include triterpenoids and polysaccharides (Kao et al, 2012). This study aims to determine ethanol extract of lingzhi mushroom on apoptotic activity and caspase-3 expression in Oral cavity cancer cells.

## 2 MATERIALS AND METHODS

### 2.1 Ethical clearance

Ensuring that the research is conducted in a responsible and ethically accountable way leads to beneficial outcomes. The ethics committee approved this research's ethical clearance, Faculty of Medicine, Universitas Jenderal Soedirman with registered number 335/KEPK/VIII/2019.

### 2.2 Preparation of *Ganoderma Lucidum* Ethanol Extract

Extraction of *Ganoderma Lucidum* fungi by maceration with 96% ethanol. Maceration involved soaking plant and material in a stoppered container with a solvent and allowed to stand at room temperature for a minimum of 3 days with frequent agitation (Azwanida, 2015). The fungus is thinly sliced and dried using an oven at 70°C for 2 hours, then mashed using a blender to become a powder. The powder was put in a 500 ml beaker and put into 96% ethanol, the ratio between the powder and the solvent was 1:5 then stirred and closed tightly with aluminium foil. The soaked powder was left to stand for 3 x 24 hours. The filtrate obtained was put into a rotary evaporator at 50°C until a thick extract was obtained and weighed.

### 2.3 Preparation of Test Extract and Control Solutions

For preparing the extract, ethanol was used as a solvent to obtain pharmacologically active compounds from the mushroom (Kumar et al, 2018). Ethanol extract of 2 mg *Ganoderma Lucidum* body fungi was dissolved with 1 ml of DMEM containing 10 µl DMSO. Solution with a concentration of 500

µl/ml was obtained. The test solution was then diluted once to obtain a concentration of 250 µl/ml, then used in serial dilutions for treatment group to obtain a concentration of 500; 250; 125; 62.5; 31.25; 15,625 and 7.8 µg/ml.

### 2.4 Culture Activation of Oral Cavity Cancer Cell (Kb CCL17)

Freezing is the most effective method of maintaining a stable supply for various cell types for long term storage (Miyamoto et al, 2018). The isolated cells were taken from the liquid nitrogen tank and diluted in a water bath with a temperature of 37°C for 12 hours and sprayed with 70% alcohol. Then the cells were put into a centrifuge tube containing 10 ml of DMEM-serum medium (DMEM was added with 10% FBS, Penicillin Streptomycin 3% and Fungizone 1%) in a laminar airflow room, then was centrifuged for 10 Minutes at a speed of 1200 rpm, Then the supernatant was removed, and the sediment that was formed was added with DMEM-serum then left to stand for 20 minutes. The cells were again centrifuged at 1200 rpm for 10 minutes, and the supernatant was removed, leaving 1 ml for resuspension. The cell suspension was inserted in a tissue culture flask (TCF) with a growth medium containing 20% FBS and was observed under an inverted microscope. The living cells looked round, shiny and clear. TCF was incubated in an incubator at 37°C and 5% CO<sub>2</sub> for 24 hours with the lid loosened.

### 2.5 Culture Harvesting of Oral Cavity Cancer Cell (KB CCL-17)

Cells were taken from the CO<sub>2</sub> incubator and harvested after 80% confluent using Trypsin-EDTA 0.25%. The media was discarded with a sterile Pasteur pipette, and the cells were washed twice with PBS. Next, 50 µL of Trypsin-EDTA added as much as 50 µL was added evenly over the cells, and then the cells were incubated again for 2 minutes. Trypsin inactivation was carried out by adding 2-3 ml of DMEM-serum, trypsin is a serine protease, was applied to cells to separate them from each other and the underlying substratum so that they can be transferred to a different vessel for re-plating (Sharma et al, 2019) after then the cells were transferred into a sterile canal. The cells were counted on a hemocytometer.

## 2.6 Immunocytochemistry Assay

Immunostaining in the process of detecting specific antigen-antibody interaction and an indirect method using secondary antibody tagged with various labels such as enzyme is commonly used (Kim, 2016). Cells were distributed into the chamber slide as much as 100 µl with a density of  $2 \times 10^4$  in each well and were incubated for 24 hours in a 5% CO<sub>2</sub> incubator to adapted and stuck to the well. Each well then was added with 100 µl of the test extract solution with a concentration of 500; 250; 125; 62.5; 31.25; 15,625 and 7.8 µg/ml. then was incubated again for 24 hours. Control used to control media in the form of a mixture of 100 µl of culture medium with 100 µl of cell suspension and 100 µl of DMSO. The preparation was then soaked in a peroxidase blocking solution at room temperature for 10 minutes. The preparations were incubated in the prediluted blocking serum at 25 O C for 10 minutes. Then it was soaked in 25OC anti-caspase 3 (NCL-CPP32p) monoclonal antibody for 10 minutes. The preparations were washed with phosphate-buffered saline (PBS) for 5 minutes. Incubation of preparations with secondary antibody (biotin-avidin)

At 25 OC for 10 minutes. Furthermore, the preparations were washed with PBS for 5 minutes. Furthermore, the preparations were incubated with peroxidase at 25°C for 10 minutes. Then the preparations were washed with PBS for 5 minutes. The preparations were incubated again with chromogen Diaminobenzinidine (DAB) at 25°C for 10 minutes and with Hematoxylin Eosin for 3 minutes. The preparations were washed, cleaned, and dripped with mounting media (Canada balsam) and terminated by closure with a coverslip under the running water. The preparations were observed under a light microscope at 200x magnification. Positive protein expression results were stained with a brownish nucleus and cytoplasm, and cells without protein expression were stained violet-blue. The active p53 protein was in the cell nucleus, while the Bax and caspase 3 proteins were in the cytoplasm (Prokhorva et al, 2018). The count of stained cells was expressed as a percentage.

## 3 RESULTS

This research began with a cytotoxic test of the ethanol extract of the Ganoderma Lucidum fungi, which will be used as the treatment group (TG) and cisplatin as the positive control group (PCG). The method used in the toxicity test of oral cavity cancer

cells in KB CCL-17 was the MTT Assay method. The results were read on 96 well-plates using an ELISA reader to obtain data in the form of optical density. The absorbance results showed that the living cells could react to the reagent to create a colour change in MTT. The results of the MTT Assay can be seen in Table 1.

Table 1: Inhibition percentages of KB CCl 17

No	Groups	Concentrations (µg/ml)	Cell Inhibition rate
1	TG1	1.95	53.94%
2	TG2	3.9	59.21%
3	TG3	7.8	63.08%
4	TG4	15.62	64.13%
5	TG5	31.35	67.42%
6	TG6	62.5	71.52%
7	TG7	125	77.49%
8	TG8	250	86.52%
9	TG9	500	92.38%
10	TG10	1000	89.45%
11	PCG1	1.56	8.89%
12	PCG2	3.125	18.26%
13	PCG3	6.25	24.09%
14	PCG4	12.5	58.41%
15	PCG5	12.5	84.4%
16	PCG6	50	94.33%
17	PCG7	100	95.11%
18	PCG8	200	70.85%

Table 1 presented the data as percentage inhibition of cells on every Ganoderma Lucidum fungi ethanol extract (TG) concentration and Cisplatin (PCG). The data used to calculate the IC<sub>50</sub> value. The IC<sub>50</sub> value was a compound parameter with cytostatic properties that inhibits cancer cells' growth by 50% were obtained from the probit analysis of the percentage of cells inhibition using the Probit Table so that the IC<sub>50</sub> value was obtained. The IC<sub>50</sub> value of the Ganoderma Lucidum fungi ethanol extract (TG) obtained at 8.49 µg/ml and cisplatin at IC<sub>50</sub>= 11.55 µg/ml (PCG). However, IC<sub>50</sub> of Ganoderma Lucidum extract on oral cancer cells from recent studies by Syairah et al (2017) was 310 µg/ml, on HL60, K562, and SGC-7901 cells were 440 µg/ml, 390 µg/ml and 900 µg/ml (Chen, 2016). Different IC<sub>50</sub> in every study has commonly occurred, although the MTT-dependent IC<sub>50</sub> errors analyzed in this study were focused on the system consisting of Ganoderma Lucidum extract, cisplatin, and oral cancer (KB CCL-17), the obtained knowledge concerning the reasons for the inconsistency in IC<sub>50</sub>

values is of practical importance for many other chemotherapeutic agents and cancer systems. Indeed, regardless of the agents or type of cancer cell lines involved, the uneven proliferation of the control cells at different seeding densities variations will yield systemic errors in IC<sub>50</sub> measurements because all of the MTT analogue assays rely on the OD reads from the control cells for the IC<sub>50</sub> calculations (Haris et al, 2016; Hafner et al 2016).

Each treatment's IC<sub>50</sub> value becomes the standard for determining the concentration dose for the Caspase-3 expression test. The treatment group of ethanol extract of lingzhi mushrooms on the expression of caspase-3 in oral cavity cancer cells were three concentrations below the IC<sub>50</sub>, namely 8.49, 4.24, and 2.12 µg/ml while the cisplatin group was one concentration IC<sub>50</sub>, namely 11.55 µg/ml.

The results of the data on the expression of caspase-3 can be seen in Figure 1

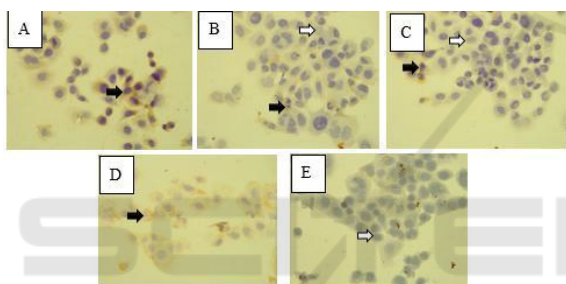


Figure.1. Caspase 3 expression on each group

The expression of caspase-3 in Figure 1 was brown, while normal cells are blue. The caspase-3 expression was calculated by dividing the number of positive cells by the number of all cells and multiplying by 100 per cent with Image J software analysis to obtain the average caspase-3 percentage.

The research data was carried out normality test using the Shapiro-Wilk test in this study was not generally distributed of 0.00 ( $p < 0.05$ ), therefore the data was transformed with log<sub>10</sub> so that a significant result was obtained of 0.19 so that the data was normally distributed ( $p > 0.05$ ). The homogeneity test was carried out. A significant value of 0.56 ( $p > 0.05$ ) was obtained. The data's variance was homogeneous and could be continued to the parametric test—the One Way ANOVA parametric test was carried out. The results of the One Way ANOVA test, the expression of caspase-3 on KB CCL-17 cells between the ethanol extract treatment groups of lingzhi mushroom (*G. Lucidum*) concentrations of 8.49, 4.24 and 2.12 µg/ml with the cisplatin group and control had high significant differences with a p-value of 0.000 ( $p < 0.01$ ). Furthermore, the post hoc Least

Significance Difference (LSD) test was carried out, aiming to determine the difference in the average percentage of the caspase-3 expression in each group. The Post Hoc LSD test results showed a highly significant difference in the percentage of caspase-3 expression in each treatment group with the control group. The difference in each group was highly significant because of the significance value was  $< 0.01$ .

## 4 DISCUSSION

The apoptotic process that arises in cells is mediated by a molecule called caspase. Caspase is a molecule that functions to carry out apoptosis in cells. Caspase can be divided into initiation groups and execution groups. Caspase 3 is an example of the caspase execution group. Caspase is activated in the extrinsic (death ligand) and intrinsic (mitochondrial) cell pathways (Mc Arthur & Kile, 2018). The zymogen form caspase 3 is necessary because if it is not regulated, caspase activity will kill all cells. In this study, the expression of caspase-3 was strongly expressed in the cell cytoplasm by showing a brownish colour. Strongly expressed in the cell was supported by the research that the apoptosis process in KB cells is likely to be induced via extrinsic pathways through caspase-3 activation in the cell cytoplasm (Hutomo et al, 2014).

The apoptotic activity and expression of caspase-3 in this study frequently increased with the addition of the ethanol extract concentration of lingzhi mushrooms related to the content.

Of lingzhi mushrooms which have an anti-cancer effect. Lingzhi mushroom (*G. Lucidum*) contains triterpenoids, polysaccharides, and ganoderic acids known to cause DNA damage effects on cancer cells to trigger apoptotic signals cells that are exposed to lingzhi mushroom extract (Wu et al, 2013; Gurovic et al, 2016). The content of polysaccharides in fungi plays a role in decreasing the mitochondrial membrane's permeability (Tian et al, 2016). This decrease causes cytochrome c to exit the mitochondria into the cytoplasm (Kole et al, 2011). Cytochrome c in the cytoplasm then binds to Apaf-1 which can activate procaspase 9 to become caspase 9. Caspase 9 will activate procaspase 3 to become caspase-3 which acts as an effector in carrying out apoptosis in cells (Ponde et al, 2019).

Caspase-3 can enter the nucleus through the pores that have been made by caspase-9, removing the substrate that causes DNA degradation. In the nucleus, there are skeleton components in the form of

lamin A and fodrin. The breakdown of lamin by caspase-3 will cause chromatin condensation, while the breakdown of fodrin triggers the formation of apoptotic bodies (Ponde et al, 2019; Zhao et al, 2020).

The chemotherapy agents' administration using cisplatin in this study obtained the highest average expression of caspase-3, of 9.7. Chemotherapy is mostly via an apoptotic mechanism that involves many proteins and genes. The most important proteins are p53 and caspase-3 (Moningka, 2019).

## 5 CONCLUSIONS

The use of herbal materials as anti-cancer therapy aimed at reducing the destructive effects of using chemotherapy agents. Lingzhi mushrooms as herbal plants are known to suppress cancer growth through the apoptotic mechanism. Further research needed regarding the effect of ethanol extract of lingzhi mushroom in inducing various apoptotic molecules.

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