The Effect of Ethanol Extract from Lingzhi Fungi (Ganoderma lucidum) Cianjur Isolate on E-Cadherin Expression KB CCL-17 Oral Cell Cancer

Pengaruh Ekstrak Etanol Jamur Lingzhi (Ganoderma lucidum) Isolat Cianjur terhadap Ekspresi E-cadherin KB CCL-17 Sel Kanker Mulut

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ABSTRAK

Kanker rongga mulut/karsinoma sel skuamosa rongga mulut (OSCC) yang berasal dari epitel rongga mulut bersifat invasif dan bermetastasis. Salah satu protein yang berperan dalam menjaga adhesi sel dalam pembentukan kanker adalah E-cadherin. E-cadherin memiliki peran penting dalam embriogenesis, berfungsi sebagai molekul penekan tumor invasif. Pengobatan yang dilakukan terhadap kanker yang invasif dan bermetastasis adalah kemoterapi dan radioterapi, tetapi pengobatan ini memiliki efek samping yang tidak diinginkan. Untuk itu diperlukan pengobatan alternatif, seperti pengobatan menggunakan bahan-bahan alami. Ganoderma lucidum (G. lucidum) diyakini dapat meningkatkan ekspresi E-cadherin sebagai anti kanker alami dan memiliki kemampuan sitotoksik dan anti-angiogenik, menginduksi apoptosis, merangsang respon imun dengan meningkatkan level limfosit CD3, CD4, dan CD8, dan merupakan antioksidan. Tujuan penelitian ini adalah mengetahui ekspresi E-cadherin pada kultur OSCC (KB CCL-17) yang diberikan ekstrak etanolik isolat G. lucidum Cianjur. Penelitian ini merupakan penelitian eksperimen dengan desain post-test only dan kelompok kontrol. Kelompok perlakuan menggunakan ekstrak etanol G. lucidum dengan konsentrasi 2,12 µg/mL (P1), 4,24 µg/mL (P2), dan 8,49 µg/mL (P3). Kelompok kontrol positif menggunakan Cisplatin dengan konsentrasi 11,5 µg/mL (K1) dan kelompok kontrol negatif menggunakan akuades (K0). Ekspresi E-cadherin diamati dengan pemeriksaan imunohistokimia. Uji ekspari E-cadherin tertinggi pada kelompok perlakuan dengan konsentrasi ekstrak etanol G. lucidum 8,49 g/mL. Uji one-way ANOVA menunjukkan perbedaan yang nyata (p<0,05) antara K0 dan P2, K0 dengan P3, K1 dengan P2, K1 dengan P3, P1 dengan P2, P1 dan P3. Ekspresi E-cadherin pada kultur karsinoma sel skuamosa (KB-CCL-17) dapat dipengaruhi oleh ekstrak etanol isolat G. lucidum Cianjur pada konsentrasi 2,12 g/mL, 4,24 g/mL, dan 8,49 g/mL.

Kata kunci: E-cadherin, Ganoderma lucidum, KB CCL-17

ABSTRACT

Oral cancer/oral squamous cell carcinoma (OSCC) originating from the oral epithelium is invasive and metastasized. One protein that plays a role in maintaining cell adhesion in cancer formation is E-cadherin. E-cadherin has an important role in embryogenesis, acting as a tumor invasive suppressor molecule. Treatment committed against invasive and metastatic cancer is chemotherapy and radiotherapy, but these treatments have undesired side effects. Therefore, an alternative treatment is desperately needed, such as that using natural materials. Ganoderma lucidum (G. lucidum) is believed to increase E-cadherin expression as a natural anticancer and has the cytotoxic and anti-angiogenic ability, induces apoptosis, stimulates the immune response by increasing the level of CD3, CD4, and CD8 lymphocytes, and is an antioxidant. The study aimed to determine the expression of E-cadherin in OSCC culture (KB CCL-17) treated by an ethanolic extract from Cianjur isolate of G. lucidum. This study was an experimental study with a post-test only with a control group design. The treatment group used ethanol extract of G. lucidum at a concentration of 2.12 µg/mL (P1), 4.24 µg/mL (P2), and
Introduction

Carcinoma is an epithelial lesion out of growth control, as shown by severe dysplasia extending through the full thickness of the epithelium with an extension of the rete pegs into the underlying lamina propria (i.e. invasion across the epithelial basement membrane), with local invasion and eventual lymphatic and blood spread, leading ultimately to metastasis to lymph nodes, and organs (mainly liver, brain and bones). More than 90% of oral cancer/carcinoma is oral squamous cell carcinoma (OSCC).  

The OSCC is the most common malignant epithelial neoplasm affecting the oral cavity. Some OSCCs arise in apparently normal mucosa, but others are preceded by clinically obvious premalignant lesions, especially erythroplakia and leukoplakia. Usually, OSCC presents as an ulcer with fissuring or raised exophytic margins. It may also present as a lump as a red lesion (erythroplakia), as a white or mixed white and red lesion, as a non-healing extraction socket, or as a cervical lymph node enlargement, characterized by hardness or fixation.  

The OSCC should be considered where any of these features persist for more than two weeks. It affects any anatomical site in the mouth and usually arises from a pre-existing potentially malignant lesion and occasionally de novo; but in either case from within a field of preconceived epithelium, OSCC had an incidence in 2018 of 354,864 cases with a mortality rate of 177,384 deaths. The increased incidence is influenced by poor prognosis, low survival rates, and poor radiotherapy treatment.  

Cancer can be invasive and metastatic which accounts for 90% of causes of death. Prediction of invasiveness and metastases can be used through protein and gene expression so that it can be a reference in determining the prognosis of tumors. In OSCC tissues, alpha B-crystallin, tropomyosin 2, myosin light chain 1, heat shock protein 27 (HSP27), stratifin, thoredoxin-dependent peroxide reductase, flavin reductase, vimentin, rho GDP-dissociation inhibitor 2 (rho GDI-2), glutathione S-transferase Pi (GST-pi), and superoxide dismutase [Mn] (MnSOD) were significantly over-expressed. Various predictors of cancer cells can be used for the analysis of oral squamous cell carcinoma, namely MMP-2, P53, and E-cadherin. The p53 protein acts as a guardian of genomic integrity that protects and prevents cells from transforming into malignant ones. Matrix metalloproteinase-2 (MMP-2) can be found in some malignant tumor growths, while E-cadherin shows low protein levels in cases of tumor malignancy. E-cadherin also has a role in tumor development by showing low protein expression in OSCC, associated with clinical and histopathological traits such as metastases, recurrence, low survival, and poor tumor differentiation and leads to a high-risk marker of malignancy.  

Qualitative and quantitative changes and function of cell adhesion molecules mediated by E-cadherin are a marker of metastases such as gastric cancer, colorectal cancer, squamous cell skin cancer, breast cancer, and oral cancer. Changes in the interaction of cells and cells with the matrix provide the ability of

Keywords: E-cadherin, Ganoderma lucidum, KB CCL-17
cancer cells to cross normal tissue lines and metastasize.\textsuperscript{7}

E-cadherin has an important role in embryogenesis such as forming tissues during gastrulation, neurulation, and organogenesis. Suppression of E-cadherin expression will result in dysfunction of intercellular adhesion. Loss of E-cadherin that occurs in precancerous lesions will accelerate the transformation into cancer, this is the beginning of changes and increases the progression of cancer cells. Cancer treatment generally uses surgical therapy combined with radiotherapy and or chemotherapy. Side effects from radiotherapy/chemotherapy treatments have disadvantages for patients,\textsuperscript{8} therefore alternative treatments are needed to minimize the side effects of radiotherapy/chemotherapy. \textit{Ganoderma lucidum} (\textit{G. lucidum}) is a fungus that has anticancer properties by inducing apoptosis, suppressing invasive cancer cells, regulating key signaling molecules,\textsuperscript{9} inhibiting the progressive cell cycle, suppressing angiogenesis and cytotoxic ability.\textsuperscript{10} \textit{G. lucidum} extract inhibited cell migration, as determined by wound healing assays, and this effect was attributed to the upregulation of E-cadherin in a cell, which plays an important role in cell adhesion processes and is downregulated in many tumor types.

Research on the effect of ethanol extract of \textit{G. lucidum} Cianjur isolates on the expression of E-cadherin as a tumor invasive suppressor molecule on OSCC KB CCL-17 has never been done before, so the authors are interested in bringing this title into the author's research material

\textbf{Methods}

\textbf{Extract}

This research is \textit{true experimental} with \textit{post-test only with control group design} against KB CCL-17 oral cavity cancer cells to the treatment group was given the ethanol extract of \textit{G. lucidum} concentration at 2.12 (P1), 4.24 (P2), and 8.49 (P3) \textmu{g}/mL. The positive control group was treated using cisplatin at a concentration of 11.5 \textmu{g}/mL (K1). The negative control group was given aqua dest (K0). The number of repetitions of each treatment and control was 4.

The \textit{G. lucidum} fungus used came from the CV. Asa Agro Corporation and was harvested after 4 months of planting and 3 months after the fruit body comes out. The fungus lived in the lowlands with a temperature of 30\degree C with a humidity of about 80\% and pH 7, taking time in September 2019. The extract used 96\% ethanol solvent using the method maceration. The fungi part used was the fruit body in which 300 g of it were thinly sliced and dried using an oven at 70\degree C for 2 hours. The ratio of powder to solvent was 1:5 (300 g in 1,500 mL of ethanol) stirred and tightly closed with aluminum foil allowed to stand for 3x24 hours. The extract was stirred and filtered daily.\textsuperscript{10}

\textbf{Cell culture}

KB CCL-17 cancer cells were originated from ATCC with epithelial morphology. The cells were taken from liquid nitrogen tanks then thawed with a 37\degree C water bath. The cells were inserted into centrifuge tubes containing 10 mL DMEM-serum medium and centrifuged for 10 minutes at 1200 rpm. The supernatant was removed and the precipitate formed was added to DMEM and allowed to stand for 20 minutes, then centrifuged at 1200 rpm for 10 minutes.

The suspension was included in \textit{tissue culture flasks} (TCF) with the grower media containing 20\% FBS, and cell morphology was observed under an inverted microscope. The picture of living cells will look like cells appearing round, shining, and clear. TCF was incubated in an incubator at 37\degree C and 5\% CO\_2 with the lid not made tense. Cells were deemed 80\% confluent, which was characterized by a yellowing medium and the cells covered the bottom area of TCF.

\textbf{Immunohistochemistry}

The cells were extracted and incubated for 24 hours on a 24 well microplate. The sample in the well was rinsed with 500 \textmu{L} PBS, washed with 7\%
ethanol and 100% hydrogen peroxide as much as 100 µL, and allowed to stand for 10 minutes. The solution was discarded with a micropipette, then cells in the coverslip were rinsed with 500 µL PBS and the PBS suspension was taken with a 1 mL micropipette and transferred to the glass preparation. Prediluted serum blocking 100 mL was dropped and allowed to stand for 10 minutes. Staining was done using E-cadherin antibody. E-cadherin expression was assessed with positive results. The general level of E-cadherin activity was assessed semiquantitatively based on the intensity of staining under a microscope with a magnification of 400 times and 10 visual fields were randomly selected. Positive E-cadherin expression was marked in blue.\textsuperscript{7, 12}

**Results**

The results were marked as positive E-cadherin expression when cytoplasm appears blue and negative E-cadherin expression if there’s no staining. The expression of E-cadherin on immunohistochemistry staining (Figure 1) and the analysis of E-cadherin expression in OSCC KB CCL-17 culture (Table 1).

![Figure 1. E-cadherin expression on immunohistochemistry examination](image)

: cells that do not express E-cadherin

: cells that express E-cadherin

<table>
<thead>
<tr>
<th>No</th>
<th>Group</th>
<th>Average (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Without treatment</td>
<td>1.58 ± 0.37</td>
</tr>
<tr>
<td>2</td>
<td>Cisplatin 11.5 µg/mL</td>
<td>5.02 ± 0.78</td>
</tr>
<tr>
<td>3</td>
<td>Ethanol Extract of G. Lucidum</td>
<td>4.61 ± 1.49</td>
</tr>
<tr>
<td></td>
<td>concentration 2.12 µg/mL</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Ethanol Extract G. lucidum Concentration 4.24 µg/mL</td>
<td>13.89 ± 0.38</td>
</tr>
<tr>
<td>5</td>
<td>Ethanol Extract of G. Lucidum</td>
<td>17.59 ± 5.31</td>
</tr>
<tr>
<td></td>
<td>concentration of 8.49 µg/mL</td>
<td></td>
</tr>
</tbody>
</table>

The results of the E-cadherin expression in Table 1 show the mean expression of E-cadherin in the OSCC KB-CCL-17 culture of each group. E-cadherin expression was most widely seen in the treatment group of G. lucidum ethanol extract concentration at 8.49 µg/mL with an average of 17.59%. The smallest expression of E-cadherin was seen in the untreated group with an average of 1.58%. The mean expression of E-cadherin in OSCC KB CCL-17 culture with the application of G. lucidum ethanol extract was increasing proportionally with increasing concentration.

The data obtained was normally distributed and homogeneous so that it met the assumptions for parametric statistical tests. The parametric statistical test used was one-way ANOVA. A one-way
ANOVA test was used to determine whether there were differences in E-cadherin expression between more than two treatment groups.

Based on the one-way ANOVA test, it was found that the expression of E-cadherin in OSCC KB-CCL-17 culture between groups without treatment, with cisplatin application, and with ethanol extract application of *G. lucidum* 2.12 μg/mL, 4.24 μg/mL, and 8.49 μg/mL had a very significant difference with \( p \)-value \(< 0.000\). One-way ANOVA test results can be further confirmed by Post-Hoc test to identify which paired groups with significant differences uses LSD (Table 2).

### Table 2. Results of Intergroup LSD Test on E-cadherin Expression

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>K0</th>
<th>K1</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>K0</td>
<td>0.18</td>
<td>0.24</td>
<td>0.001 **</td>
<td>0.000 **</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>K1</td>
<td>0.18</td>
<td>0.855</td>
<td>0.003 *</td>
<td>0.000 **</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Q1</td>
<td>0.240</td>
<td>0.86</td>
<td>0.002 *</td>
<td>0.000 **</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>P2</td>
<td>0.001 **</td>
<td>0.000 *</td>
<td>0.000 **</td>
<td>0.120</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Q3</td>
<td>0.000 **</td>
<td>0.000 **</td>
<td>0.000 **</td>
<td>0.120</td>
<td></td>
</tr>
</tbody>
</table>

Note: * = meaningful (\( p < 0.05 \))
** = very meaningful (\( p < 0.01 \))

K0 = negative control (without treatment)
K1 = positive control (cisplatin 11.35 μg/mL)
P1 = ethanol extract of *G. lucidum* concentration 2.12 μg/mL
P2 = ethanol extract of *G. lucidum* concentration of 4.24 μg/mL
P3 = ethanol extract of *G. lucidum* concentration of 8.49 μg/mL

Based on the LSD test results (Table 2), it was found that there were significant differences in E-cadherin expression between each group except between the K0 group and K1 group, K0 group with P1 group, K1 group with P1 group, and P2 group with P3 group. The difference between each group is said to be significant if the value of \( p < 0.05 \). The \( p \)-value between the K0 group and the K1 group is 0.18, the \( p \)-value between the K0 group and the P1 group is 0.24, the significance value between the K1 group and the P1 group is 0.86, and the \( p \)-value between the P2 group and the P3 group which is 0.12.

### Discussion

Based on the results of the one-way ANOVA test, there were differences between the untreated group, the cisplatin group, and treatment groups P1, P2, P3. Ethanol extract of *G. lucidum* was able to increase the expression of E-cadherin in OSCC KB CCL-17. *G. lucidum* was used as an anticancer and can inhibit cell formation, cell migration, and spread of tumor cells in epithelial ovarian cancer cells. This may occur because *G. lucidum* inhibits the cell cycle in the G2/M phase and also induces apoptosis by activating caspase 3.13

Research conducted by Acevedo-Diaz *et al*. showed that *G. lucidum* has anticancer effects with cytotoxic mechanisms and the ability to induce apoptosis in HCT-116 colon cancer cells. The ability of apoptosis can be observed by the increased expression of Bax/Bcl-2 through the intrinsic pathway involving caspase, resulting in depolarization of the mitochondrial membrane.\(^{14}\) *G. Lucidum* can suppress viability, migration, and invasion ability in breast cancer cell culture. This is mediated by the downregulation of signals in molecules such as the activity of *rac*, *ENA/NASP*, *p-
FAK (tyr925), Cdc42, and c-Myc expression. Reduced Rac activity will reduce the ability of metastasis in breast cancer.\textsuperscript{15}

Based on this research, there is a difference in the expression of E-cadherin between the treatment of \textit{G. lucidum} ethanol extract and the group without treatment. The mean expression of E-cadherin in the treatment group was greater than in the group without treatment. This study was in line with research conducted by observing E-cadherin expression in nasopharyngeal cancer metastases. The number of positive E-cadherin expressions in cancers with distant metastases (M1) was less than those of negative E-cadherin. The decreased expression of positive E-cadherin occurs due to E-cadherin loss mediated adhesion disorders. Hence, tumor cells could separate from the primary tumor tissue which will potentially lead to metastasis.\textsuperscript{7} E-cadherin positive expression in metastasis of head and neck squamous cancer is less observed than the number of negative E-cadherin expressions. The reduced expression of E-cadherin is the opposite of the expression of cyclin D1 which increases in metastasis. The cause of the lower E-cadherin expression is associated with several gene mutations.\textsuperscript{11} Function changes of E-cadherin occur due to missense mutations in ovarian cancer. E-cadherin gene mutations are also found in diffuse gastric cancer with a percentage of 30%. The mutation will cause the cutting or loss of E-cadherin protein and changes in amino acid sequence. Mutations in E-cadherin have an atlas genome in the form of TCGA.\textsuperscript{16}

E-cadherin expression has decreased because there is no epithelial transition to mesenchymes with marked low vimentin levels in inflammatory breast cancer.\textsuperscript{4} Decreased expression of E-cadherin accordingly occurs due to a downregulation of the protein-mediated by reduced levels of eIF4G5. Extract of \textit{G. lucidum} can reduce gene expression and may affect the ability of cell proliferation and survival, invasiveness, and metastatic properties of breast inflammatory cancer cells. This is characterized by a decrease in protein expression of BCL-2, TERT, PDGFB, MMP-9, E-cadherin, eIF4G, p120-catenin, c-myc, and gelatinase activity.\textsuperscript{9} Differences of E-cadherin expression in invasive and metastatic breast cancer cells. E-cadherin decreased during invasive and increases in the event of metastasis. E-cadherin is associated with epithelial-mesenchymal transformation (EMT) in cancer cells, metastasis will be initiated from epithelial changes to mesenchymal tissue. The loss of expression of E-cadherin during the EMT is often thought to promote metastasis by allowing the dissociation and invasion of cancer cells. The loss of E-cadherin will increase the expression of Fgll, Foxj1, and Bmp2 which are indicators of good prognosis in breast cancer, besides it will reduce the expression of pro-metastasis and pro-inflammatory transcription such as Il33 and Stfa3. Furthermore, loss of E-cadherin will cause apoptosis signal induction and cell death in the invasive ductate carcinoma (IDC) model. Induction of apoptosis occurred in connection with the TNFα, TGFB, and p53 pathways after the loss of E-cadherin. This pathway will induce stress oxidative and reactive oxidative species (ROS) that regulate metastasis. Bonding between loose cells will cause ROS accumulation.\textsuperscript{6} Loss of E-cadherin expression in invasive ameloblastoma size 3.5 cm more than ameloblastoma with size <3.5 cm, this occurs because E-cadherin acts as a cell adhesion molecule, interacts with heparin with growth factors, and has an important role in regulating cell adhesion with extracellular matrix molecules.\textsuperscript{17} However, a meta-analysis study suggested that reduced E-cadherin expression might be a predictor of a poorer prognosis and could be a potential new gene therapy target for breast cancer patients.\textsuperscript{18} E-cadherin expression is not related to clinical pathology parameters, but a loss of E-cadherin expression in cancer determines poor prognosis in maintaining life. The loss of E-cadherin function occurs
due to modifications by *growth factors* such as *epidermal growth factor receptor* (*EFGR*), loss of EFGR expression will cause reduced E-cadherin expression because EFGR can regulate expression in cell adhesion molecules associated with cell growth and invasive ability.\(^{19}\)

Based on the above studies, the mean expression of E-cadherin without treatment showed a value of 1.58% on the CCL-17 KB cell line. Other research shows that express E-cadherin in the *cell line of gastric cancer* was 11.6% and 8.2%, on a *cell line of colon cancer* was 10.7%, in pancreatic cancer is 3.1% and lung cancer by 2%.\(^{20}\)

This experiment proved that there were differences in the mean expression of E-cadherin between the administration of *G. lucidum* ethanol extract concentration 4, 24 \(\mu g/mL\) and 8.49 \(\mu g/mL\) with cisplatin, the average amount of E-cadherin expression of *G. lucidum* ethanol extract higher than cisplatin. The results of this study indicate that *G. lucidum* ethanol extract affects increasing the number of E-cadherin expressions so that *G. lucidum* can be developed as an anticancer potential agent to reduce the level of invasive and metastatic OSCC.

**Conclusion**

Ethanol extract from *G. lucidum* Cianjur Isolate can increase E-cadherin expression in squamous cell carcinoma culture in concentration at 2.12 \(\mu g/mL\), 4.24 \(\mu g/mL\), and 8.49 \(\mu g/mL\).

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