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The Role of Sarang Semut (Myrmecodia pendans) Flavonoid's Fraction in Proliferation and Angiogenesis Inhibition of Human Tongue Squamous Cell Carcinoma

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Abstract
Background: Squamous cell carcinoma is the most common type of cancer that occurs in the oral cavity which is about 90-95 % of total malignancy in the oral cavity. The aim of this study was to analyze the effect of the flavonoid's fraction of Sarang Semut (Myrmecodia pendans) as an anti-cancer to angiogenesis inhibition by suppressing the VEGF and IL-8 expression in SP-C1 human tongue cancer cell. **Material and Method**: This research was conducted with the pure laboratory experimental method using Supri's-Clone 1 (SP-C1) human tongue cancer cell culture. This research was started with cytotoxicity test to obtain the fraction of flavonoid that possesses anti-cancer potentiality and angiogenesis inhibition test. **Result**: The result of this study showed that ethyl acetate and ethanol fraction of flavonoid had a role in inhibiting the VEGF protein and interleukin 8 expressions in SP-C1 tongue cancer cell. ANOVA test with significance level ($\alpha = 0,05$) which resulted in highly significant difference in the concentration with a mean optical density (OD) absorbance ethanol fraction ($p = 0,00$), ethyl acetate fraction ($p = 0,00$), and controls ($p = 0,00$) for the suppression of VEGF protein expression and interleukin-8 of SP-C1 human tongue cancer cell. Western blotting analysis showed that ethyl acetate fraction of flavonoid application to SP-C1 human tongue cancer cell showed a decrease in SP-C1 protein expression. **Conclusion**: Flavonoid fraction of sarang semut inhibit the angiogenesis in SP-C1 tongue cell cancer by suppressing the VEGF and interleukin 8 protein expression. Ethyl acetate fraction from sarang semut's flavonoid inhibited the expression signal transduction factor from SP-C1 tongue cancer cell.
Keywords: SP-C1 tongue squamous cell carcinoma, sarang semut flavonoid's fraction, angiogenesis, VEGF, interleukin-8.

Introduction

Cancer is one of the diseases which is characterized with disruption or failure in multiplication setting mechanism in multicellular organisms resulting in uncontrollable cellular behavioural changes. The changes are due to changes or genetic transformation, especially changes in the genes that regulate growth, namely proto-oncogene and tumor suppressor genes. The cells that undergo transformation proliferate continuously and suppress the growth of the normal cells. Cancer is a disease with high mortality rates[1]. Global Action against Cancer data (2005) from World Health Organization (WHO) states that death caused by cancer could reach 45% from 2007 to 2030 which is about 7.9 million to 11.5 deaths. In Indonesia, based on Riskesdas report (2007), cancer's prevalence was approximately about 4.3 for each 1.000 populations, and the seventh cause of death (5.7%) after stroke, tuberculosis, hypertension, trauma, perinatal, and diabetes mellitus.[2]

Squamous cell carcinoma is one of the most common types of cancer that occurs in the oral cavity, which is about 90-95% of total malignancy in the oral cavity. Squamous cell carcinoma location in the oral cavity is usually located on the tongue (ventral and lateral), lips, floor of the mouth, buccal mucosa, and retromolar area.[3] Squamous cell carcinoma of the tongue is a malignant tumour derived from epithelial mucosa of the oral cavity and is largely a type of epidermoid carcinoma. Squamous cell carcinoma of the tongue had a range between 25 and 50% of all malignant cancers in the oral cavity.[4]

Several studies have proven the efficacy of sarang semut for the cancer treatment. Based on those researches, sarang semut herbs can be used as an alternative medicine of breast cancer chemotherapy with minimal side effects. Using sarang semut as a traditional medicine, the treatment cost will be suppressed and it has a minimum side effects compared to the expensive chemotherapy which has a lot of side effects.[5] Sarang semut contain flavonoids, tannins, and polyphenols that act as an antioxidant, so that it is good for cancer prevention. In addition, sarang semut also contain tocopherol and alpha-tocopherol, a highly active substance with free radicals inhibition capability.[6] Supri's cell clone (SP-C1) has been widely studied in order to obtain the anticancer substances from medicinal plants (herbs) as well as the effectiveness of synthetic drugs on the growth of cancer cells. SP-C1 is a tongue cancer cells isolated from patients with lymphonadi, which is derived from

moderately differentiated of squamous cells and have not invaded the muscle tissues.[7]

MATERIAL AND METHOD

This research was conducted with pure laboratory design using the Supri's-Clone (SP-C1) human tongue cancer cells. The study was conducted at Laboratory of Integrated Research and Testing (LPPT) Gadjah Mada University in Yogyakarta from July to October 2013. Sarang semut originated from Jayawijaya Regency, Papua. Approximately 900 grams of fresh sarang semut was extracted using ethanol and then was evaporated to obtain the ethanol extract. Concentrated ethanol extract was dissolved in distilled water and then partitioned in a separating funnel using n-hexane to obtain the fraction of n-hexane and water (H_2O). Water fractions obtained subsequently partitioned between water, ethyl acetate, and ethanol to obtain the ethyl acetate and ethanol fraction.

Cytotoxicity assay in this study was performed by incubating the cells with a number of 2 x 104 cells for 24 hours using serial concentration of sarang semut's flavonoid. This research used MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide), a tetrazolium salt which is commonly used in the quantitative determination of living mammalian cells or cell proliferation using in vitro calorimetric method. These methods can only be used on live cells and not in dead cells because the test was based on degrees of cell activation. Sarang semut flavonoids concentration in the cytotoxicity assay with the interval number of 1000 µg/ml in the upper limit and 7.812 µg/ml in the lower limit was 7.812 µg/ml, 18.625 µg/ml, 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, subsequently, and 0 µg/ml as a control.

Measurement of the expression of VEGF and interleukin-8 on sarang semut flavonoid fraction was performed using a quantitative technique based on ELISA reader interpolation against a standard curve. The measurement using ELISA reader with λ_{max} = 450 nm produced the Optical Density (OD) data, an interval data that would subsequently processed to obtain the VEGF and IL-8 concentration and the data would be interpolated to the normal concentration in order to obtain the angiogenesis inhibition percentage. Based on this result, the data would be processed and it was expected that suppression of VEGF and IL-8 expression would be observed.

RESULT AND DISCUSSION

The result of the cytotoxicity assay showed SP-C1 tongue cancer cell death percentage in each fraction continued to increase along with the increased concentration. Ethyl acetate and ethanol fraction were the most potential cell growth inhibitor compared with the hexan and water fraction. Ethyl acetate fraction from flavonoid in a concentration of 1000 µg/ml resulted in the death of cell as much as 64.60% and the lowest concentration of 7.8125 mg/ml led to the death of cell by 15.80%. The result of the average cell death due to the exposure from four different concentrations has been presented in Figure 1.

Based on the result of the cytotoxicity test, LC_{50} in each fraction was 452.059; 937.562; 2691.535; 12302.69 µg/ml for ethyl acetate, ethanol, hexan, and water fraction, respectively. This result was obtained from the equation of the line curve vs. log concentration probit. This study referred to Meyer's Standard (1982)[8] which stated that a substance said to be active or to have toxic properties when LC_{50} values < 1000 µg/ml for the extracts and ≤ 30 µg/ml for the compound. An extract was considered toxic when the LC_{50} value was about 30-1000 µg/ml and was not considered toxic when the LC_{50} value was above 1000 µg/ml. The toxicity level was used to describe the significance from the extract's potential activity as an antitumor. LC_{50} was used as a parameter to identify the potential cytotoxicity of sarang semut's flavonoid fraction to the SP-C1 tongue cancer cell. The smaller the LC_{50}, the more toxic compound. Based on the LC_{50} values from ethyl acetate fraction of flavonoid (452.059 µg/ml) and the ethanol fraction of flavonoid (937.562 µg/ml) which was below 1000 µg/ml, we could conclude that ethyl acetate and ethanol fraction of flavonoid from the sarang semut had a cytotoxic activity to the SP-C1 tongue cancer cell which was based from the Meyer's criteria (1982).[8]

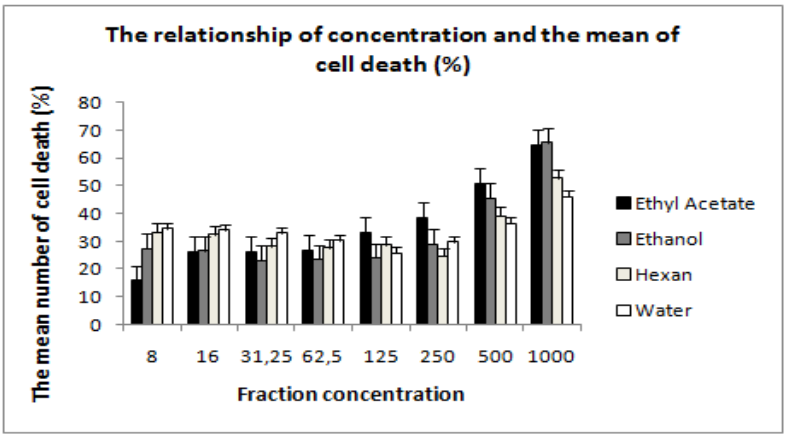

Figure 1. The cytotoxic effect of ethyl acetate, ethanol, hexan, and water fraction from sarang semut (Myrmecodia pendans) to the SP-C1 tongue cancer cell.

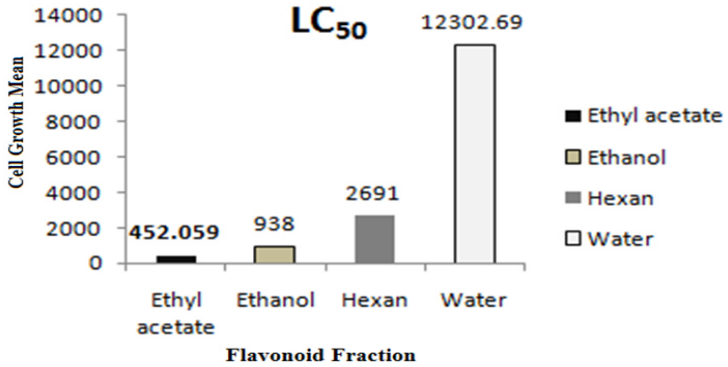

Figure 2. LC_{50} value from each flavonoid fraction in the cytotoxicity test.

The measurement of VEGF and IL-8 expression on the sarang semut flavonoid fraction was conducted using ELISA reader quantitative technique which was based on the interpolation to the standard curve. The measurement used the ELISA reader with $\lambda = 450$ nm and produced Optical Density (OD) data, an interval data which would be used to obtain the VEGF and IL-8 concentration and would be interpolated to the normal concentration, so that angiogenesis inhibition percentage would be obtained. Based on these result, the data will be analyzed and it was expected that the expression of VEGF and IL-8 would be suppressed. Based on Figure 3 and 4, we could see the same pattern in the inhibition of VEGF and IL-8 expression which was interpreted as the greater the concentration of the flavonoid and ethanol fraction, the inhibition of protein expression would be greater. In contrast to the untreated SP-C1 cell (control), the protein expression of VEGF and IL-8 were increased. The direction of the line drawings in the figure showed that the concentration level of VEGF and IL-8 expression was up to 15.625 µg/ml in SP-C1 that showed the similar levels of the protein, but after the concentration reach the value of 31.25 µg/ml, the protein of VEGF and IL-8 showed a different level or there was a decreasing concentration. The result of this research showed a statistically significant difference ($p = 0.000$).

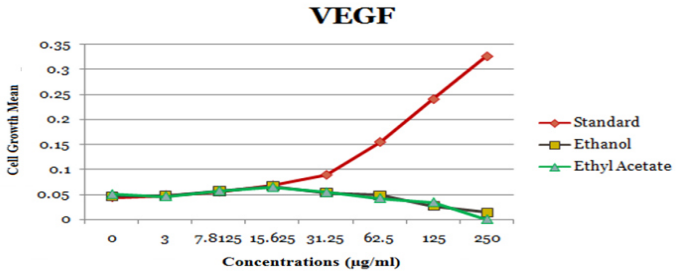

Figure 3. The result of ethanol and ethyl acetate fraction inhibition test to the protein of VEGF and IL-8 expression of SP-C1 tongue cancer cell.

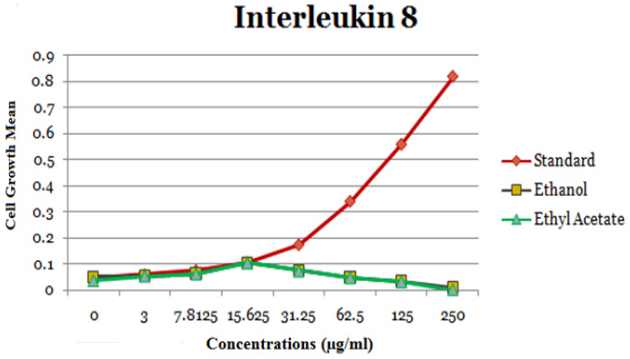

Figure 4. The interleukin-8 expression to the SP-C1 tongue cancer cell post-treated with the ethyl acetate and ethanol fraction of flavonoid.

The result of this study was in line with the result from Tan et al (2002) study about quercetin inherited in *G. Procumbens* flavonoid's leaves extract could suppress the tumor growth in vitro and in vivo by tyrosin kinase inhibition activity and angiogenesis growth factor, VEGF, by inhibiting the new vascular formation.[9] Some of the new research had also explain about the flavonoid contents from *G. procumbens* extract may inhibit the VEGF receptor (VEGFR) by inhibiting the matrix metalloproteinases (MMP) activity, tyrosin kinase, and cyclooxygenase-2 (COX-2).[10] Bachelder et al (2001) had confirmed the VEGF role in cancer cell survival by using antisense oligonucleotide method to reduce the VEGF expression.[11] This research used breast cancer cell in serum media 10%. By reducing the VEGF expression for about 50%, cancer cell apoptosis would be significantly increased. Hasan et al (2011) in his research using the colon cancer cell observed that by inhibiting the autocrin VEGF could increase the cell senescence process. Senescence occurred when cell cycle had stopped permanently, so that the cancer cell experienced the regression.[12]

Other research confirmed the theory about the relationship of protein component that play a role in cell cycle by angiogenesis process. Gupta et al (2006) in his research showed the interaction between E2F1 transcriptions factor with p53 could affect the angiogenesis process by directly regulating VEGF transcription.[13] On the other hand, Claudio et al (2000) observed that pRb2/p130 (family pRb) could regulate the angiogenesis by inhibiting VEGF in G1 phase.[14]

This research was supported by Gibbs (2000) in his research which used the ethanol extract from *B. gymnorhiza* skin stem showed a probability in using natural molecular compound such flavonoid as an anticancer.[15] The first probability, these natural compounds could inhibit the signal transduction in cell membrane. Signal transduction was started from outer cell signal such growth factor which acknowledged by the receptor. The receptor would transmit the proliferative signal to the cytoplasmic protein. These signal transduction activated by phosphorilation process using ATP and protein, generally the kinase proteins. Signal transduction cascades process could be inhibited by some of the natural compound which contained kinase inhibitor compound, so that it could inhibit the signal transduction cascades, including the flavonoid.

The second probability, it could affect the cell cycle programs by inhibiting the cell cycle progression and inducting the cell cycle arrest. The tested compound could inhibit the pRb phosphorilation or inducting the INK4 family proteins, a CDK inhibitor protein. This cell cycle progression inhibition could also occur in the S phase, G2/M transition which usually observed as a cell cycle arrest.[16]

Molecular mechanism that caused the cell cycle arrest using flavonoid was still unknown. Flavonoid could change the DNA binding from the transcription of nuclear factor kappa B (NF-κB). NF-κB could be inducted or overexpressed in some of the cancer cell, and its activation could induce cell proliferation and malignant transformation with differentiation and apoptosis. Cancer cell intervention using flavonoid could cause NF-κB binding to the DNA which later caused the apoptosis.[18]

Flavonoid as an anticancer medicine could reduce the COX-2, so that prostaglandin production would prevent.[19,20]

CONFLICT OF INTERESTS

This research has not received any specific fund. The authors declare that they have no conflict of interests.

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