Ethanol Extract of Borneo's Bajakah Tampala Root (*Spatholobus littoralis hassk*) Supresses the Cell Proliferation and Chemotactic Migration and Induces Apoptosis on A Human Oral Tongue Squamous Cell Carcinoma Cell

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Abstract

Oral tongue cancer is characterized by high degree of proliferation, local invasion and a high rate of metastasis to the cervical lymph nodes. At the time being, the root of the Bajakah tampala has attracted keen interest.

The aim of study was to investigate the effect of ethanolic extract of Bajakah tampala root toward a human oral tongue cancer (H357 cell). A study on the cell proliferation, chemotactic migrated and apoptosis induction. The MTT assay was confirmed to detect the suppression of cell proliferation. The inhibition of cell chemotactic migration was delivered by Boyden chamber kit assay. To evaluate the apoptosis induction was examined by double staining acridine orange-ethidium bromide (AO-EB). The concentration of extract of Bajakah tampala was set as 0, 10, 25, 50 and 100 μg/mL. Data were analyzed by Two-way ANOVA followed by LSD with the level of significance at 95%.

Results of study revealed extract of Bajakah tampala at concentration 50 to 100 μg/mL was markedly suppressed the proliferation and chemotactic migration cell, and significantly induced apoptosis cells (P<0,05). Conclusion, Extract of Bajakah tampala has potential for strong antitumor activity against human oral tongue cancer cells through inhibition of cell proliferation, migration, and induction of apoptosis.

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Introduction

Bajakah tampala root is a plant that lives in the tropical rain forests of Borneo, Indonesia, which is currently popular because it has potential as an anticancer drug. The root of Bajakah is daily made for tea drinks by the Borneo's people. It has the full name bajakah tampala (*Spatholobus littoralis hassk).* To date, there are 29 species of the genus *Spatholobus Hassk*, most of which are found in the tropical forests of Indonesia and Malaysia.[1] Bajakah root was known to have efficacy as an anticancer drug and used by Borneo's residents as a natural medicinal plant. All parts of this herbal plant to be used as medicine for dysentery, aches and pains, infections, inhibiting bleeding and wound medicine. Bajakah tampala has secondary metabolites in the form of phenolic compounds, flavonoids, tannins, and saponins. These saponins and tannins can stimulate the process of angiogenesis, an important part of the wound healing.[2] Furthermore, Bajakah tampala root was also reported it has rich antioxidant content, even thousands of times more than other types of plants that have been found, especially for the treatment of human cancer. It was reported that a concentration of 10% ethanol extract of Bajakah tampala root was more effective in healing incision wounds than concentrations of 20% and 40%.[1] However, the studies of Bajakah tampala root in the oral cancer are no reports.

Oral cancer including tongue cancer is a malignancy that ranks tenth in men worldwide. Tongue cancer has a rapid cell growth, high invasion and metastasis, and is categorized as

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malignancy with high recurrence.[3-6] There is a tendency to increase the occurrence of tongue cancer, especially in the anterior two thirds of the tongue and there is also an increase in cases of tongue cancer in young patients (under the fifth decade). Tongue cancer is the most common malignancy in the oral cavity and is characterized by rapid aggressive biological behavior. The 5-year survival rate in patients with tongue cancer has been reported to be lower than 50% over the past 40 years. These conditions are very different from the significant increase in survival rates in patients with solid tumors in other parts of the body.[7] The incidence of oral cancer varies between countries, and generally correlates with exposure to tobacco-derived carcinogens, excessive alcohol consumption, or both. Tongue cancer that occurs in the oral cavity is strongly associated with previous infections including oncogenic human papillomavirus (HPV) strains, especially HPV-16, HPV-18 and other strains.[8]

Based on the aggressive nature of oral cancer and the high ability of cell growth, invasion and metastasis, we tried to explore an alternative antitumor drug using the root of Bajakah tampala specifically from the tropical rain forests in Central Borneo, Indonesia, which has been empirically known to be a natural medicine for various diseases including human cancer. The purpose of this study was to analyze the antitumor activity of the ethanolic extract of Bajakah tampala root against human oral tongue squamous cell carcinoma (H357). Focus study on the inhibition of proliferation and chemotactic migration, and induction of cell apoptosis.

Materials and methods

Cells and cell culture

Tongue cancer cells (H357: ECACC General Cell Collection, UK) were cultured in Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich, USA) supplemented with 10% fetal calf serum (FCS, Moregate BioTech, Australia), 100 µg/mL streptomycin, and 100 units/mL penicillin (Invitrogen Corp., USA). H357 cells were incubated at 95% humidity and 5% CO_2 at 37^0C.

Preparation of Bajakah roots extract and dilution of concentrations

Bajakah root extract was prepared using the maceration method with the following stages: the Bajakah tampala root was prepared by making small pieces with a size of 0.5 cm. The pieces of Bajakah tampala root was dried using an incubator at 55^0C for 72 hours. The smooth root pieces to become powder (simplicia) by blending it with a blender machine. The powder was mixed with 70% ethanol in a ratio of 1:1 (weight/volume). The mixture of simplicia and solvent was homogenized using a shaker at 150 rpm for 72 hours. The solution was filtered using Whatman filter paper number 41 with a diameter of 110 mm. Concentrate the filtrate using a vacuum rotary evaporator. A solution of Bajakah tampala root extract was prepared by dissolving the extract using a suspending agent in accordance with the test concentration. A stock solution of Bajakah root extract with 1 gr/mL concentration was diluted into 10, 25, 50 and 100 µg/mL in a solution of DMEM 10% FBS. These concentrations were incubated with H357 cells for 24 and 48 hours.

Cell Proliferation assay (MTT assay)

Oral tongue cancer (H357) cells were cultured on 96-well plates (Falcon, USA) at a rate of 2.0×10^4 cells per well in DMEM 10% FBS, the day before. All cells were incubated with various concentrations of ethanol extract of Bajakah root (0, 10, 25, 50 and 100 µg/mL). After 24 and 48 hours, cell numbers were quantified by MTT: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazoliumbromide (Sigma-aldrich)]. Absorption measurements were detected by a BioRad microplate reader (BioRad, USA) with a wavelength of 550 nm.

Cell chemotactic migration assay with Boyden chamber apparatus

Chemotaxis (directed migration) was evaluated with a Boyden chamber apparatus (Neuro Probe, Inc., Cabin John, MD, USA). Briefly, sub confluent cells for 24 hours were harvested with 0.05% (w/v) trypsin (Invitrogen Corporation, USA) containing 0.02% (w/v) ethylenediamine tetra-acetic acid (EDTA, Invitrogen Corporation, USA). Subsequently, it was washed twice with PBS, and resuspended to a final concentration of 5×10^5 per mL in serum-free medium with 0.1% (w/v) bovine serum albumin fraction (BSA, Wako Pure Chemical Industries, Ltd). A polyvinyl-pyrrolidone (PVP) filter (Nuclepore Corp, Palo Alto, CA, USA) with a pore size of 8 µM was coated with gelatin (Merck KGaA, Frankfurt, Darmstadt, Germany) (0.1 mg/ml) and rinsed with sterile water. The bottom chamber was filled with 30 µL DMEM 10% FBS

plus various concentrations of Bajakah root extract, and covered with a gelatin-coated PVDF membrane. Furthermore, 50 µL of cell suspension, which was equivalent to 500 cells/mL was introduced into the upper chamber. After 24 h of incubation, the membranes were stained with Giemsa's solution (Ted Pella Inc., Redding, CA, USA). The number of cells that had passed through the filter was counted under a light microscope at 400x magnification. Calculations were carried out in 12 fields in each concentration.

Apoptosis Induction using double staining (AO-EB) analysis

Apoptotic induction was analyzed by acridine orange (AO) and ethidium bromide (EB) double staining analysis. Briefly, equal amounts ($2x10^4$) of cell substrates prepared from H357 cells were cultured in 6 cm diameter well-covered well plates. Then, they were incubated with various concentrations (0, 10, 25, 50 and 100 µg/mL) for 24 hours. After incubation, the media was aspirated and washed with sterile PBS. AO-EB solution at 3 mL was added to each test well and incubated at room temperature for 10-15 minutes. Each cover glass is placed on the object glass upside down and immediately examined under a fluorescence microscope. The results were photographed as document. Each test performed triplicate.

Statistical analysis

Statistical differences between the means of the different groups were evaluated with Stat View 4.5 (Abacus Concepts, Berkeley, CA) using two-way ANOVA and *t-test*. The significance level was set at 5% for each analysis.

Results

Cell growth inhibition (MTT Assay)

The results of the study on the antitumor activity of the ethanolic extract of the root of Bajakah tampala (Spatholobus littoralis hassk) against human tongue squamous cell carcinoma (H357) through studies of proliferation, migration chemotactics and induction of cell apoptosis have been completed. Determination of the analysis of inhibition of H357 cell proliferation was carried out using the MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) which was incubated for 24 and 48 hours.

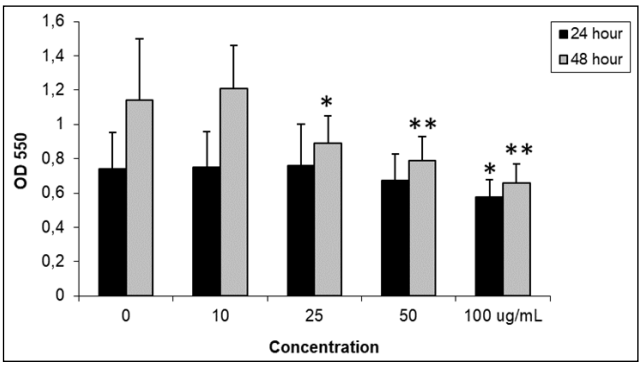

Figure 1. The growth inhibition assay of tongue cancer cells (H357) were incubated using various concentrations of Bajakah tampala root extract for 24 and 48 hours. (*: P<0.05 and **:P<0.01, Two-way Anova).

Based on Figure 1, it is known that in the 24-hour incubation period the ethanol extract of Bajakah tampala root revealed an increase in the growth of H357 cells to a concentration of 10-25 µg/mL compared with control, after that from a concentration of 50 to 100 µg/mL there was a cell growth inhibition of 9.1% to 22.4% (P<0.05). Furthermore, during the 48-hour incubation period, it was found that H357 cell growth inhibition occurred from a concentration of 25-100 µg/mL of 21.9% to 42.2% (P=0.00).

Migration Chemotactic Test (Boyden Chamber Assay)

The inhibition of chemotactic activity on the migration of oral tongue squamous cell carcinoma (H357 cells) incubated with various concentrations of Bajakah tampala root extract was delivered by Boyden chamber kit for 24 hours. The results of the migration test showed that there was a very significant difference between the concentrations of the Bajakah tampala root extract on the inhibition of migration chemotactic activity (P = 0.00).

Based on Figure 2, it can be seen that healthy cells undergo chemotactic migration with round, clear cell shapes and an evenly distributed thin layer. Meanwhile, cells that undergo apoptosis or necrosis appear to have a thick black layer of cells that are not evenly distributed, or even the cells are completely black.

Figure 3 revealed the migration chemotactic inhibition of H357 cell at an extract concentration of 10-25 µg/mL known to be 19.6% to 33.3%, while for a concentration of 50-100 µg/mL it was found to be 47.1% to 65.2% with IC50 at an extract concentration of 76.7 µg/mL.

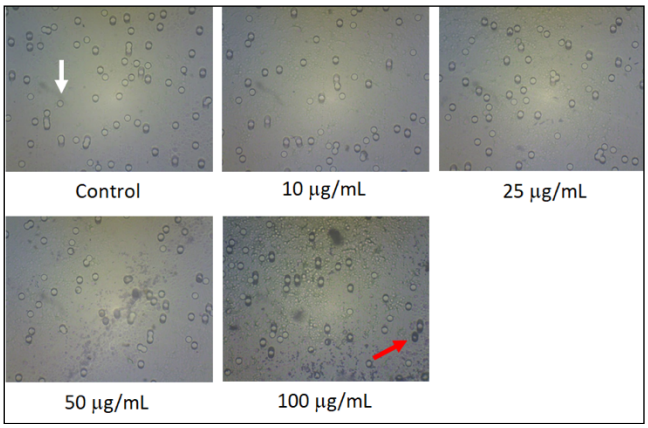

Figure 2. Chemotactic morphology of H357 cell migration incubated at various concentrations of Bajakah Tampala root extract. (white arrow: healthy cells migrate; red arrow: apoptosis or necrosis cells).

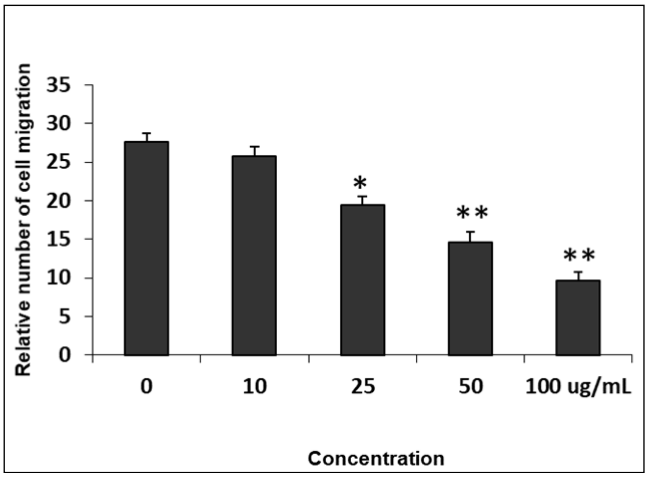

Figure 3. The relative number of H357 cells migrated after being incubated with various concentrations of Bajakah tampala root extract for 24 hours. (*: significant, $p<0.05$; ** very significant, $p< 0.01$).

Induction apoptosis (Double staining analysis AO-EB)

Based on double staining analysis using Acridine Orange-Ethidium Bromide, it was found that there was significantly increased of apoptosis in H357 cells incubated with ethanol extract of Bajakah tampala root according to the increase in the concentration of the extract, especially at high concentrations in this study (50-100 µg/mL). Based on Figure 4, it was known that a concentration of 50 µg/mL induces apoptosis at 36.5% within a 24 hour incubation period, and concentration of 100 µg/mL was found to increase apoptosis of H357 cells by 61.7%.

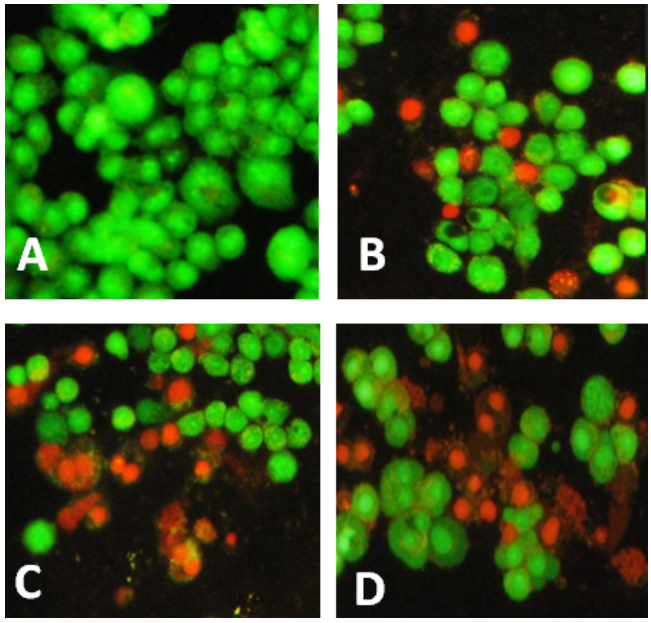

Figure 4. Induction of apoptosis of human tongue cancer cells (H357) induced by Bajakah tampala root extract for 24 hours. A. Control, B. concentration 25 µg/mL, C. concentration 50 µg/mL, and D. concentration 100 µg/mL.

Discussion

The focus of researchers in the field of oral cancer is currently increasing cell growth inhibition, chemotactic migration or cell invasion, metastasis and apoptosis induction.[9] Proliferation, invasion and metastasis of cancer cells and resistance to therapy are important phenomena in the malignancy and progression of cancer cells. Abnormal regulation of cell cycle proteins and an imbalance between dead cells and living cells is one of the factors that trigger the occurrence of malignancy and aggressiveness of cancer cells.[10] In this time, conventional treatments for oral cancer such as radiotherapy, chemotherapy, surgery and their combination, continue to give unsatisfactory results.[4] Similarly, research on inhibition of chemotactic cell migration, proliferation, invasion, metastasis, and induction of apoptosis in relation to human tongue cancer using natural ingredients Bajakah tampala root has not yet been reported and this study is the first to report it.

The Bajakah tampala root came from the interior of the tropical rain forest of Central Borneo (Kalimantan Island).[1] The roots were extracted using ethanol as a solvent in order to attract polar, semi-polar and non-polar compounds because ethanol has properties as a

magic solvent. Bajakah root was known to have efficacy as an anticancer drug and used by Borneo's residents as a natural medicinal plant. Bajakah tampala root was also reported it has rich antioxidant content, even thousands of times more than other types of plants that have been found, especially for the treatment of human cancer.[1,2] In the cell proliferation assay, it was found that at 24 hours of incubation in the extract solution, the inhibition was 9.1% to 22.4% from a concentration of 50–100 µg/mL. But a small concentration showed an increase in cell proliferation. These results indicate that these concentrations were able to increase the expression of tumor promoting genes or proteins (p38Jab1 and p45Skp2), while the high concentrations indicate an increase in the expression of tumor suppressor genes or proteins (p27Kip1 and p21Waf1).[4,11] Furthermore, the incubation time of 48 hours was known to have inhibition of 21.9% - 42.2% from a concentration of 25-100 µg/mL. These data indicate that there was a strong inhibition of the proliferation of human oral tongue cancer cells. The results of this study were strengthened by the strong increase in chemotactic migration suppression of human oral tongue cancer cells at concentrations of 50-100 µg/mL. The important result was that the IC50 in this migration assay was known to be 76.7 µg/mL. This concentration was very small and indicates that Bajakah tampala root extract is very strong in inhibiting the migration or invasion of human oral tongue cancer cells compared to the proliferation assay. In addition, the greater concentration of the extract in this chemotactic migration assay shows the greater inhibition of cell migration activity. It was reported that intercellular proteins like integrins and cadherins play a role in cells migration.[12] If the expression of both proteins is low, the cell migration will increase. In this study it was detected that the Bajakah root extract was able to strongly inhibit the chemotactic migration activity, it is possible that the expression of the intercellular protein of oral cancer cells was also increased. Furthermore, the same results were shown in the apoptosis induction analysis using acridine orange-ethidium bromide double staining.

It was known that the greater concentration of the extract revealed the greater induction of cell apoptosis, especially at high concentrations in this study (50-100 µg/mL). These data showed that Bajakah tampala root extract has potential for strong apoptosis induction as well as cell chemotactic migration assay. It was reported that imbalanced regulation of apoptosis contributes greatly to the occurrence of various diseases including cancer, restenosis, stroke, heart failure, neuro-degenerative diseases, and AIDS.[13] It was also reported that molecular machinery is responsible for the occurrence of apoptosis through the family of intracellular protease caspases, and that the morphological and biochemical changes of cells are characteristic of the phenomenon of apoptosis.[14,15] The most important outcome of this experiment was this study is the first time to be published.

Conclusions

In conclusion, ethanol extract of Bajakah tampala root has strong antitumor activity against human tongue squamous cell carcinoma cell proofed by inhibition of cell proliferation and chemotactic migration as well as induction of cell apoptosis.

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Declaration of Interest

The authors report no conflict of interest.

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