Apoptosis Induction (Caspase-3,-9) and Human Tongue Squamous Cell Carcinoma VEGF Angiogenesis Inhibition using Flavonoid's Ethyl Acetate Fraction of Papua Ant Hill (*Myrmecodia pendans*) SP-C1

Harun Achmad[1], Supriatno[2], Sri Ramadhany[3], Marhamah Singgih[1], Rasmidar Samad[4], M. Hendra Chandha[5], Sri Oktawati[6], Hendrastuti Handayani[1]

1. Pediatric Dentistry Department, Hasanuddin University, Makassar, Indonesia.
2. Oral Medicine Department, Gadjah Mada University, Yogyakarta, Indonesia.
3. Public Health Department, Hasanuddin University, Makassar, Indonesia.
4. Dental Public Health Department, Hasanuddin University, Makassar, Indonesia.
5. Oral Surgery Department, Hasanuddin University, Makassar, Indonesia.
6. Periodontology Department, Hasanuddin University, Makassar, Indonesia.

Abstract

Human tongue squamous cell carcinoma was casued by inability to control cell survival and to control cel motility. Apoptosis is a normal component process of multicellular organism's development and health. Apoptosis is a programmed cell death as a response to various stimuli. Angiogenesis or neovascularisation is a basic developing sign in cell physiology and pathology.
This research was to identify flavonoid's ethyl acetate fraction from Anthill (Myrmecodia pendans) which has a potential as a anti-cancer to SP-C1 cell.

This research was to analyze the Anthill flavonoid's ethyle acetate as an anti cancer to apoptosis induction (caspase-3 and -9 analyses) to Supri's Clone (SP-C1) tongue cancer cell and to analyze angiogenesis inhibition by VEGF protein expression. The data from this research was analyzed using Two Way ANOVA and t test (Tukey's test) with confidence interval 95%.

Caspase-3 and -9 colorimetric analyses showed increasing SP-C1 cell inhibition by time and concentration. ELISA analysis showed increasing proteolytic activity on caspase-9 compared with caspase-3. Increasing proteolytic activity with the increasing concentration from 5 µg/ml to 100 µg/ml could also be observed. VEGF angiogenesis inhibition showed increased flavonoid concentration was followed by increased VEGF expression. Otherwise, SP-C1 cell (control) did not show any VEGF expression.

Anthill's flavonoid ethyl acetate fraction (Myrmecodia pendans) had anti-tumor activity on several molecular targets from apoptosis and VEGF angiogenesis of tongue squamous cell carcinoma.

Experimental article (J Int Dent Med Res 2018; 11(1): pp. 276-284)

Keywords: SP-C1 tongue cancer cell, Anthill plant, Apoptosis, Angiogenesis, VEGF.

Received date: 14 September 2017 **Accept date:** 01 December 2017

Introduction

Cancer is a disease that comes from a group of abnormal cells that grow and develop in the process of cell division. Carcinogenesis, which begins with a number of cells that follow abnormal signals to divide and differentiate cells continuously. This is caused by DNA mutations that produce proteins that disrupt cellular balance. This signal autonomythen develop these cells, so that growth being uncontrolled and proliferation occured. This proliferation if left continuously will spread into cancer cells.[1,2]

The distribution of tongue cancer in Indonesia is 1.01% of all cancer cases and 42% of all cancers of the oral cavity with male and female ratio is 1.65: 1. The frequency of oral cavity carcinoma tends to increase and until now has been ranked 6th of 10 most common cancer found in developing countries.[1] The lateral area of the tongue has an incidence of 25% of the total number of squamous cell carcinomas in the body and 50% of all squamous cell carcinomas in the oral cavity.[1,2]

High cell proliferation and uncontrolled nature due to interruption of protooncogene

***Corresponding author:**
Harun Achmad
Pediatric Dentistry Department,
Hasanuddin University, Makassar, Indonesia.
E-mail: harunachmader@gmail.com; febyr.yuferaa@yahoo.co.id

factor and tumor suppressor genes balance resulting in increased production of growth factors and the number of cell surface receptors that can stimulate intercelluler signal transduction to increase the production of transcription factors. DNA damage causes cessation of cell cycle at the G $_1$ phase and then repair process will occur, if the DNA damage cannot be repaired then these cells will undergo apoptosis.[3]

*Supri's-Clone*cell (SP-C1) has been studied to obtain anticancer compounds from medicinal plants (herbs) and the effectiveness of synthetic drugs on the growth of cancer cells. SP-C1 is a tongue cancer cell isolated from lymph node in tongue cancer, derived from a moderately differentiated squamous cell carcinoma and has not experienced an invasion to muscle tissue. SP-C1 cells have rapid growth characteristics, rapid invasion and rapid metastasis, inflammatory diseases that hard to recover, very high recurrences despite radical surgery had been done and patient's shorter duration of life. [3,4]

Cancer occurs because of changes in the physiological basis of cells that can grow into malignant cells with common characteristics, such as not being sensitive to anti-growth signals, avoiding apoptosis, having unlimited replication potential, angiogenesis, invasion and metastasis to other tissues. Therefore, the target of anti-cancer drug development is directed to induction/pace apoptosis.[3,4]

The etiologic factors of tongue cancer in children are still debated. Factors suspected as being a factor in the etiology of tongue cancer are genetic factors, previous viral infections, and immunodeficiency states. Socioeconomic status and oral hygiene are the predisposing factors in causing tongue cancer in children.[4,5]

Several tissue culture trials have shown that in cancer there is an increased activity of Ras oncogene pathways that trigger proliferation and increased Akt pathways that suppress apoptosis.[5,6] Apoptosis is programmed cell death, an important process in the regulation of normal cell proliferation homeostasis, this process produces a balance in the number of specific tissue cells through the elimination of damaged cells and physiological proliferation, so the tissue function remained normal.[7] Apoptosis acts to maintain in order to retain normal tissue function. Deregulation of apoptosis results in pathological conditions, including uncontrolled cell proliferation as seen in cancer.[7,8] One of the functions of apoptosis is to prevent the occurrence of cancer by eliminating preneoplastic and neoplastic cells (accretion of abnormal new cells).[7,8] Control of apoptosis associated with genes that regulate the cell cycle, such as the gene *p53,* Rb, myc, E1A, and *bcl-2* family. Apoptosis occurs if internal monitors detect any damage dysfunction and gave the signal to begin a series of programs (cascade) which in turn activates the cysteine aspartate protease protein (caspase) and endonucleases to kill cancer cells. Apoptosis regulation is to maintain normal homeostasis, maintaining the balance of proliferation and cell death in multicellular organs.[7,8]

Cancer therapy that currently done such as removal of cancer tissue and chemotherapy, still felt not effective. The removal of cancer tissue is usually done and generally cannot completely eliminate the cancer because there is a possibility of tissue that still left behind and can grow into new cancer tissue.[9] While chemotherapy and irradiation are less selective in killing cancer cells, often normal cells also break down and die. Therefore it is necessary to seek a relatively safe cancer treatment.[10,11]

Anthill's flavonoid represent a very diverse class of about 9000 secondary plant metabolite structures.[12] Flavonoids are polyphenol compounds derived from 2-phenylchromane commonly found in many plants, vegetables, and flowers. Flavonoids received attention in the literature, particularly because of the biological and physiological characteristics in the health field that is anti-oxidant, metal chelation, anti-proliferative, anti-cancer, anti-bacterial, anti-inflammatory, anti-allergic, and have anti-viral effects.[12,13]

From the results of chemical screening tests, known that anthill plants contain phenolic chemical compounds of flavonoid groups. [14] Flavonoids are natural antioxidants conducted as a hydroxyl radicals, superoxide and peroxyl radicalsreductor. Inhibiting the progression of cancer was suspected because of *Myrmecodia pendans* that contained flavonoids. Flavonoids are polyphenols compound that are known as important components in human diet. Flavonoids have been shown to inhibit the development of cancer cells by stimulating the production of interferon-gamma (IFN- ɣ) in immunocyte populations.[14,15]

Topoisomerase enzyme inhibitors will stabilize the topoisomerase complex and cause the DNA to be cut and damaged. Damaged DNA can lead to the expression of proapoptotic proteins such as Bax and Bak and decrease the expression of antiapoptosis proteins, Bcl-2 and Bcl-XL. Thus the growth of cancer cells is inhibited. Most flavonoids have been shown to inhibit the proliferation of various cancer cells in humans but are non-toxic to normal human cells.[16]

Apoptosis has a role in the physiological processes of cellular autodestruction that important for the development, homeostasis and multicellular organism hostsdefense. Apoptosis is a part of the normal physiological development of the body which is divided into 3 phases, namely the induction phase, the effector phase, and the degradation phase. [17] In the induction phase depends on the signal that cause of death, and stimulate proapoptotic signals and initiate a cascade. The signal that cause of the deaths such as reactive oxygen species excessive activation from Ca^{2+} pathways, protein families of B-cell lymphoma 2 (Bcl-2) such as Bcl-2-associated x protein (Bax) and Bcl-2-associated death promoter (Bad). In the effector phase, the cell will become death at the regulating center, ie the mitochondria toward to cell death. [18] The last phase is the degradation phase involves a serial events that occur both in the cytoplasm and in the nucleus of cells. Caspase activation occurs within the cytoplasm whereas in the cell nucleus occurs chromatin condensation, the nuclear shell breaks and DNA fragmentation occurs to subsequently become apoptotic body that being phagocytosis by surrounding cells as well as by macrophages.[17,18]

At the molecular level apoptosis is divided into 3 phases, namely the initiation phase, the execution phase and the termination phase. In the initial phase, apoptosis stimulated by various factors such as the low concentration of the growth factor, gamma radiation, chemotherapy drugs, and signals from death receptors. The execution phase is characterized by bubbles of cell membrane *(blebbing),* nucleus fragmentation, chromatin condensation and DNA degradation. In the termination phase apoptotic body will being phagocytosis by phagocyte cells so that the cells will become lysis. Apoptosis occurs through two paths triggered by internal and external factors. Apoptosis through internal factors called intrinsic pathway or mitochondrialpathwayswhile through external factors called extrinsic pathway (death receptor pathway*)*.[19]

Caspase is anapoptosis main mediator key necessary for tissue development and homeostasis. There are 100 caspase substrate and 12 caspase proteases sub class that have been identified, namely caspase 1-12. Proteases are important mediators to degrade proteins and recyclingprotein. Caspase is synthesized in an inactive precursor form called procaspase. The proteolytic process of procaspase produces an active tetrameric caspase enzyme. Based on kinetic data, substrate specificity and procaspase structure, conceptually caspase is differentiated into caspase initiator and caspase effector.[20]

The caspase initiator plays a role in activating the caspase effectors in response to specific cell death signals. The procaspase initiator is activated by oligomers whereas the caspase effectors are usually activated by other proteases that almost all of them were caspase initiators, as well as by other proteases through trans-activation. *In vitro,* it has been known that procaspase 3 and procaspase 7 can be activated by caspase 6, 8, 9, 10. Caspase 3, 6 and 7 areboth direct or indirect effector caspase. [21] Caspase-9 is an essential component of aspartic acid cysteine proteases. After stimulation of apoptosis, cytochrome crReleased from mitochondria. This complex is processing a 9-procaspase into small, medium, and large active fragments. To get the caspace-9, it found on the intrinsic pathway of apoptosis.[22]

Vascular Endothelial Growth Factor *(VEGF)* is a growth factor that stimulates angiogenesis, are divided into direct and indirect angiogenic molecules. VEGF belongs to a direct angiogenic molecule. VEGF is also known as vascular permeability factor (VPF) is the most important factor proangiogenic and most widely expressed in various types of tumors, both benign and malignant tumor cells. VEGF derived from growth factors family specifically targeted endothelial cells to increase permeability of the endothelial cells through the signal transduction cascade of mitogen-activatedprotein kinase (MAPk) by loosening the connection between endothelial cells in the cadherin complex*.* Termination of vascularization is important to start angiogenesis because it causes some proteins such as metalloproteinase matrices (MMPs) deposited in extracellular fluids. MMPs break down the extracellular matrix to allow migratory endothelial cells and invade areas adjacent to cancer.[23] Hypoxia that occurs in the cancer cells growth caused by oxidative stress which then leads to the inflammatory state. Hypoxia that occurs in cancer cells will activate hypoxia inducible factor-1 (HIF), which stimulates VEGF. VEGF is a growth factor that will initiate angiogenesis process.[23,24] This research was to identify flavonoid's ethyl acetate fraction from Anthill (*Myrmecodia pendans*) which has a potential as a anti-cancer to SP-C1 cell. This research can also be used as a scientific base to use herbal medicine such as Anthill Flavonoid to inhibit human tongue squamous cell carcinoma in terms of cellular and molecular biology.

Materials and methods

This research was conducted at Research and Development Laboratorium (LPPT) Faculty of Dentistry, Gadjah Mada University, Yogyakarta, Indonesia. The research was conducted from April, 3rd-2017 to May, 30th-2017. This research was funded from Research Grand Program, Ministry of Research, Technology, and Higher Education of the Republic of Indonesia.

Activation of Cell Line Tongue Cancer SP-C1Cells

Tongue cancer SP-C1 cell was taken from the tank of liquid nitrogen and then thawed in a water bath at 37 ^{0} C until melted and then sprayed with 70% alcohol. Cell was inserted into centrifuge tubes containing 10 ml of medium DMEM (Sigma-Aldrich, USA) plus 10% FBS (Gibco, Brooklyn, MA, USA), penicillin, streptomycin 3% (Penstrep; Gibco, USA), and 0.5% fungizone in the laminary airflow (Thermo scientific, USA), and was centrifuged (Sakura Seiki Co. Ltd., Japan) at 1200 rpm for 5 minutes. The supernatant was removed, the precipitate formed was added with DMEM (Sigma-Aldrich, USA) serum. After being left for about 20 minute the cells were centrifuged at 1200 rpm for 5 minutes. Supernatant removed. The cell suspension was put into the TFC (Tissue Culture Flask) with the growth media containing 10% FBS (Penstrep; Gibco, USA) and viewed under an inverted microscope (Olympus, Japan).

Breeding of cancer Cells SP-C1 Tongue

Cancer cells in a flask then centrifuged at 2000 rpm for 5 minutes. Supernatant removed and left about 1 ml for re-suspension. After a homogenous cell suspension, added DMEM media (Sigma-Aldrich, USA) containing 10% FBS (Penstrep; Gibco, USA) and then the cancer cells are distributed into multiple TFC (Tissue Culture Flask). In laminary airflow (Thermo scientific, USA) the old media discarded and the attached cells then sprayed slowly with new media. The cell suspension obtained were distributed into several flasks, stored in an incubator (Sanyo Electric Biomedical Co. Ltd., Japan) at 37 ^{0} C, 5% CO_2.

Cell proliferation inhibition (MTT assay)

In testing the proliferation inhibition, put up 3 plates containing 24 wells, MTT assaytest with MTT; 3- (4,5 dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide (Sigma-Aldrich, USA) at 0, 24, 48, 72 hours. Then insert tongue cancer SP-C1 cell on each plate as much as 2×10^4 cells / wells in 100 μL of DMEM (Sigma-Aldrich, USA) in accordance with the concentration of flavonoids compound. Based on the calculation, the total number of cells required is 12.8×10^5 cells for whole wells and the amount of DMEM solution (Sigma-Aldrich, USA) required is 25.6 ml. The calculation of the number of cancer cells is determined using a hemocytometer (Hausser Scientific, Pennsylvania, USA). All cells were then incubated at 37˚C for 24 hours. Plate of 24 wells is measured with a Bio-rad Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA) OD with a wavelength of 540 nm. Tested at 0, 24, 48 and 72 hours

Apoptotic Test SP-C1 (Caspase-3 and Caspase-9 Calorimetric Analysis)

The apoptosis method was conducted in 2 ways, namely the coloring technique and the calorimetric analysis of caspase-3 and -9. Staining was done with acridine orange and ethidium bromide. The calorimetric analysis used 2 plate ofs 96-well, for caspase -3 and -9 testing. The number of cells required for apoptosis detection procedure is $2x10^4$ cells / wells. Each of the wells that being contained by cells are added with a 3 compound medium solution with a concentration under the IC_{50} for each compound and incubated for 24, 48 and 72 hours. Cells that have undergone apoptosis are centrifuged at

1500 rpm in a conical tube for 10 minutes. The supernatant was discarded and the cells lysed by the addition of 25 μL of $1x10^4$ cells Lysis Buffer. Cell lysates were incubated on ice pack for 10 minutes then centrifuged at 10,000 rpm for 1 minute. The supernatant was transferred into a new tube and kept on ice packs. The cell lysate was placed into a 50 μl / wellplate of96wells. Each wells on the first plate, added with 5 μL caspase-3 colorimetric substrate (DEVD-pNA), a second plate was added with 5 μL caspase-9 colorimetric substrates (LEHD-pNA) and incubated for 1-2 hours at 37^0 C. Plate was read using a microplate reader (ELISA reader) with a wavelength of 405 nm

Angiogenesis Test

The VEGF standard solution was manufactured according to the manufacturer's instructions. Prepared plate of 24 wells that have been coated with VEGF-specific monoclonal antibody (already included in the kit). The two leftmost wells were filled with 0.1 ml of standard solution no. 3.[23] The last two wells are filled with the diluent buffer as the control (Zero wells). Plate then incubated in a water bath for 90 minutes at 37° C. After 90 minutes, the contents discharged without washing plate and added with 0.1 ml VEGF biotinilated antibody into each wells. Plate was incubated again for 60 minutes. Plate washed again with 0.01M PBS 5 times, each washing time allowed to stand for 1-2 minutes. Washing solution is discarded after each wells added with 90 mL TMB dye (3,3 ', 5' - tetramethylbenzidine) and incubated for 15-20 minutes. The blue color was seen in 4 standard solutions with the highest VEGF concentration. After 20 minutes, each well was added with 0.1 ml of TMB stop solution. The color changinginto yellow was seen instaneous. OD read using a microplate reader (BioRad, USA) at a wavelength of 450 nm immediately after the stop solution given. The data from this research was analyzed using Two Way Anova and t test (Tukey's test) with confidence interval 95%.

Results

Cell proliferation inhibition (MTT assay)

The result of proliferation inhibition test showed that at the 0th hour, cell growth rate stay still, it was the control group to see that the cells growth in each plate of 96 wells had the same cell growth rate before inhibited by anthill ethyl acetate fraction flavonoid. At 24 hours the cell growth inhbition begins at concentration 25, as well as at 48 and 72 hours. However, the magnitude of the inhibition is different, the biggest inhibition occurs at 72 hours. With increasing concentrations, at 24, 48 and 72 hours shows an increasingly large inhibition, especially at 72 hour at a concentration of 100 μM, large inhibition can be seen. A 125 μM concentration in all time groups showed anincreasing growth rate compared to a concentration of 100 μM. This shows that at such concentrations are not effective in inhibiting cell growth. This result was depicted in Figure 1.

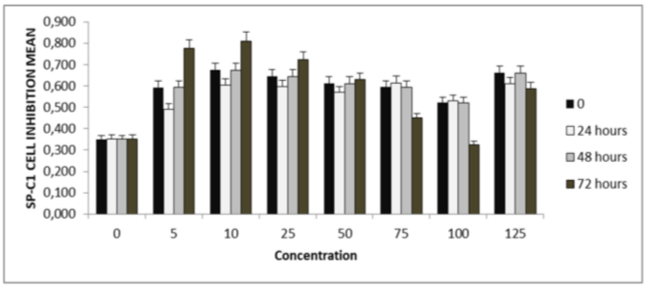

Figure 1. Profile of tongue cancer SP-C1cell growth proliferation assay results from anthill ethyl acetate fraction at time 0, 24, 48 and 72 hours.

SP-C1 Apoptosis Test (Caspase-3 and Caspase-9 Calorimetric Analysis)

The test Results on apoptosis test using a acridine orange and ethidium bromide coloring by observing the number of cells undergoing apoptosis using fluorescent micro scope resulting green living cells, whereas cells undergoing apoptosis become yellow (Figure 2). Cells that do not undergo apoptosis becoming green fluorescence due to the cancer cells do not absorb the acridine orange dye and unable to absorb ethidium bromide because the integrity of the cell that are still good.

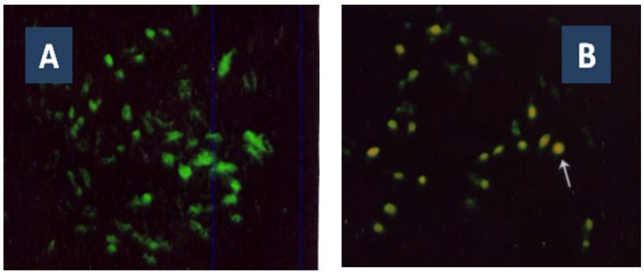

Figure 2. Double staining apoptosis. EB (ethidium bromide) - AO (acridine orange) with Caspase-9 (A = live cells, B = apoptosis cell).

In the calorimetric analysis, observing the apoptosis induction of SP-C1 human tongue cancer cells treated with anthill ethyl acetate fraction flavonoid in several concentrations ie, 5, 10,25,50,75,100 and 125 µg / ml within 24 and 48 hour. By using colorimetric analysis of caspase-3 and -9 observed an increasing activity of caspase-3 and -9 for each concentration studied. Controls for each group based on the time span of the study, ie 24, 48 and 72 hours. The observed of treated samples within 24 hours appear through a light microscope (100x magnification) showed dead cells floating on the surface of the media. This shows the apoptosis induction of human tongue cancer SP-C1cells. Changes seem to begin at the concentration of 5 µg / ml anthill ethyl acetate fraction flavonoid in the presence of dead cells. The number of dead cells continues to rise to peak at concentration of 100 µg / ml, and decreases at concentration of 125 µg / ml.

Observation results that being treated within 24, 48 and 72 hours showed an increasing dead cells to a concentration of 100µg / ml anthill ethyl acetate fraction flavonoids. Treatment effects began to show at the concentration of 5 µg / ml anthill ethyl acetate fraction flavonoids that increased to peak at a concentration of 100 µg / ml, and decreased since concentration of 125 µg / ml.

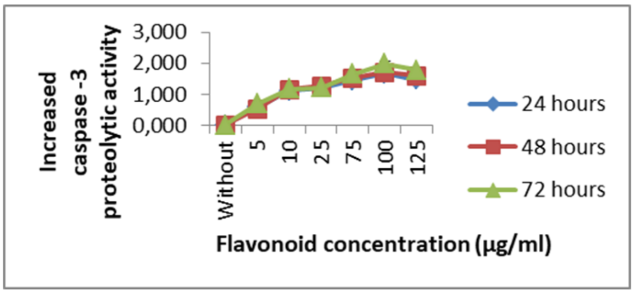

Figure 3. Increased multiples caspase-3 proteolytic activity for span of 24, 48 and 72 hours (by 4.5 times), * p<0.05.

Increased caspase-9 proteolytic activity was significant for each concentration based on Two Way Annova test as shown in Figure 4 (p < 0.05). The highest peak of activity occurred at concentrations of 100 µg / ml (4 times increasing at 24 and 48 hours and 4.5 times increasing at 72 hours) began to decrease at concentrations of 100 µg / ml. This data indicates that the most effective caspase-9 activity after treated with anthill ethyl acetate fraction flavonoids was at a concentration of 100 µg / ml over a 72 hour time span.

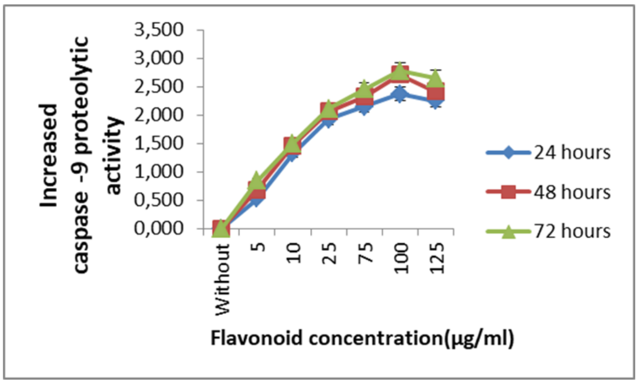

Figure 4. Increase in multiples of proteolytic activity of caspase-9 span of 24, 48 and 72 hours (by 4.5 times), * p <0.05.

A significant increasing of caspase-3 proteolytic activity for each concentration over different time spans, 24, 48 hours (4-fold increase) and 72 hours (4.5-fold increase) as shown in Figure 3 (p < 0,05). The highest peak activity occurs at concentrations of 100 µg / ml over a 72 hour time span. These data suggested that the most effective caspase-3 activity after treated with anthill ethyl acetate fraction flavonoids is at a concentration of 100 µg / ml over a 72 hour time span.

Angiogenesis test (VEGF protein expression)

Analysis of angiogenesis inhibition test through the VEGF expression inhibition were performed using SPSS 16.0 version for windows .From statistical analysis results using Kolmogorov-Smirnov test showed that the data were normally distributed because of greater than 0.05 (p> 0.05). The data was tested using Two Way Anova test with significance level □ = 0:05. The result is that there are very significant differences between concentrations with a mean optical density (OD) ethyl acetate fraction (p = 0.000) and controls (p = 0.000) on the suppression of VEGF protein expression of tongue cancer SP-C1cells. The results of this study indicated that the anthill ethyl acetate fraction flavonoid content has the ability to inhibit angiogenesis growth through VEGF protein inhibition expression on tongue cancer SP-C1 cell (Figure 5).

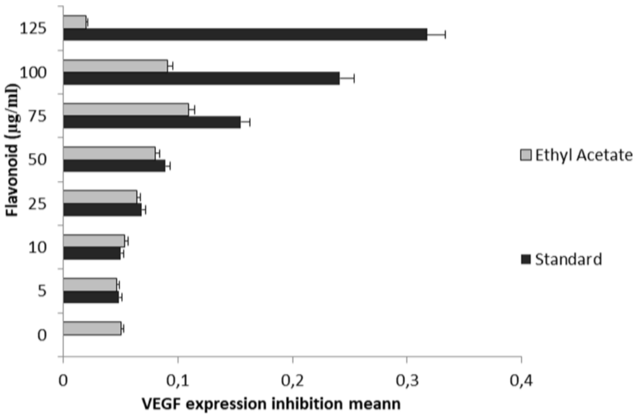

Figure 5. VEGF protein expression in tongue cancer SP-C1 cell after treated with Myrmecodia pendans' flavonoid fraction.

Discussion

Increased caspase-3 and -9 activity in this research could be observed for each concentration from 24, 48,and 72 hours, significantly, in this current research. Similar findings were reported in previous studies.[25] Apoptosis is an important mechanism for preventing cell proliferation from damaging DNA. Tumor cells that unable to undergo apoptosis is one of the underlying factors for tumor growing larger and cells genetic instability. Defective mechanism of apoptosis can also increase cell survival. Causes the malignant cells expansion regardless of the cell division process.[25]

The results of this study indicated that increased proteolytic activity on caspase-9 was higher (4.5-fold) compared to caspase-3 (4-fold) at a concentration of 100 µg/ ml in span of 72 hours. These results showed that the anthill ethyl acetate fraction flavonoid play a role in the apoptosis induction through the mitochondrial pathway (intrinsic pathway). Some research also suggested that anthill ethyl acetate fraction flavonoids affect on several stages of cell, including caspase-9 activation, release of cytochrome c, and damage the mitochondrial membrane permeability.[25,26]

One of the body mechanisms to overcome abnormalities in the cell level to keep the body in a homeostasis state is to automatically turn off the cell itself called apoptosis. This mechanism is different from necrotic cell death. Deficiency of vitamin C due to lack of food intake often occurs in cancer patients. Research has been done is low levels of vitamin C in serum malignant patients (≤11 µmol / l) and low survival rate in these patients.[25] The death of a human tongue cancer SP-C1 cells through apoptosis process expected through a mechanism of anthill ethyl acetate fraction flavonoid that can inhibit the COX-2 action through the stages: (1) Inhibition of Akt1 in activating anti-apoptotic bcl- 22power.[24] Akt1 have different kinds of influences, which one is the apoptosis inhibition through the intrinsic pathway. Mechanism of Akt1 action affect on release of cytochrome-C from mitochondria to systole, and this will be prevented by the anti-apoptotic members of gene bcl-2. Inside the cytosol,cytochrome-C along with Apotosis Protease Activating Factor-1 (Apaf-1) and pro-caspase 9 forms caspase 9, this complex is called apoptosome.[26,27,28]

Based on the different concentrations, the results of this study, there was a significant difference for each concentration level. The lowest concentration of 5µg / ml already showed an increased of caspase-3 and -9activity, and keep raising for each concentration and peaked at the concentration of 100µg / ml, after that declined in concentration of 125 ug / ml. The influence of anthill ethyl acetate fraction flavonoid action at each concentration, indicating loss of cancer cell viability. Loss of cell viability is one indicator of apoptosis induction. The results showed that at a concentration of 5 µg / ml loss of cancer cells viability has occurred and reached its peak at a concentration of 100 µg / ml.

At concentrations of 5 µg / ml the presence of proteolytic activity indicates that in this concentration the cells begin to capture and carry the substrate. Furthermore, from the results of the study the decrease of proteolytic activity began to occur at concentrations of 125 µg / ml. This decrease is related to the occurrence of saturation (saturation point) on human tongue cancer SP-C1 cells after achieving optimum concentration at 100 µg / ml. When the cell is saturated, the condition that occur in the cellular stage is that the receptors have become saturated to transport substrates that affect on apoptosis induction, which should be in the cell membrane phase.

In cancer cells there are no extracellular growth signals integration and coordination with "cell cycle regulators". Consequently there wereuncontrolled growing cell, independent on whether or not an extracellular signal can grow without a growth signal and is less responsive to inhibitory signals. One of the important cancer cells properties in culture is its lifetime with unlimited proliferation capability and abnormal differentiation. Abnormal differentiation is associated with the inability of cells undergoing apoptosis, whereas apoptosis is a differentiation program of many types of cells that have limited life survival, anthill ethyl acetate fraction flavonoid given on SP-C1 cells showed results for each concentration and could increase apoptosis in tongue cancer SP -C1cell.[29]

In the angiogenesis inhibition test showed that the flavonoid content has an important role in inhibiting the angiogenesis growth through inhibition of VEGF protein expression on tongue cancer SP-C1 cells (Figure 5). Flavonoid contained in the anthill plant *(Myrmecodia pendans)* in this study thought to suppress tumor growth *in vitro* by tyrosine kinase activity inhibition and growth factor angiogenesis which is VEGF protein expression by inhibiting the formation of new blood vessels (neovascularization). Several other studies have also explained that the anthillplant flavonoid *(Myrmecodia pendans)*has the possibility in inhibiting VEGF receptors (VEGFR2) through inhibition of activity Matrix Metalloproteinase (MMP), tyrosine kinase, and Cyclooxygenase -2 (COX-2).[29,30]

Several other studies that supported the results of this study in theory that there is a relation between protein components thatplay a role in the cell cycle with angiogenesis process. The interaction between E2F1 transcription factor and p53 can affect the occurrence of angiogenesis by regulating VEGF transcription directly. In addition, pRb2 / p130 (family pRb) regulates angiogenesis by inhibiting VEGF in G1 phase.[31,32] This research identified flavonoid's ethyl acetate fraction from Anthill (*Myrmecodia pendans*) which had a potential as a anti-cancer to SP-C1 cell. This research could also be used as a scientific base to use herbal medicine such as Anthill Flavonoid to inhibit human tongue squamous cell carcinoma in terms of cellular and molecular biology.[34,35] The limitation of this research was to achieve confluent cell's count due to temperature changes. Further biology-cellular studies would be needed in other aspects to strenghten the use of *Myrmecodia pendans*'s flavonoid fraction, such as its effect to cancer cells metastases, invation, and other inhibition effect.

Conclusions

Anthill's flavonoid ethyl acetate fraction (*Myrmecodia pendans*) had anti-tumour activity on several molecular targets from apoptosis and VEGF angiogenesis of tongue squamous cell carcinoma.

Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

References

1. Feller et al., Oral squamous cell carcinoma: epidemiology, clinical presentation and treatment. Journal of Cancer Therapy. 2012;263-8.
2. Van Der Wall, I., Epidemology and clinicopathological aspect of premalignant dan malignant lesions, in: Peckham, M., Pinedo, H., Veronesi, U. Oxford Textbook of Oncology Vol 1, Oxford University Press. 1995;985-1001.
3. Achmad, H. Akt signal transduction line and nuclear factor-kappa b transcription (nf-κb) as molecular targets of squamous cell tongue scale cells (sp-c1) using papua's anthill nest (Myrmecodia pendans). Pak. J. Biol. Sci. 2016;19: 323-30.
4. Achmad, H. Anti-cancer activity and anti-proliferation ant nests flavonoid fraction test (Myrmecodia pendans) human tongue cancer cells in SP-C1. International Journal of Dental and Medical Sciences. 2014;13(6): 2279-81
5. Rajendran, R., Sivapathasundharam, B., Shafer's textbook of oral pathology. 5th ed. Elsevier. 2006;494.
6. Bourgoyne, JR. Oral cancer. Philadelphia Lea and Febiger. 1954;62-77.
7. Buletin kanker. Kementrian Kesehatan Republik Indonesia. Jakarta. 2015.
8. Petrkovic, I. et al. Burkyt lymphoma in elderly patients. Scientific journal of the faculty medicine. 2013;30(2): 103-5.
9. Tanaka, T., Ishigamori R. Oral carcinogenesis and oral cancer chemoprevention: a review. Hindawi Publishing Corporation. Pathology Research International. 2016;394-7.
10. Achmad, H., Suryani, Supriatno, Marhamah. The role of sarangsemut (Myrmecodia pendans) flavonoid's fraction in proliferation and angiogenesis inhibition of human tongue squamous cell carcinoma. International Journal of Biology, Agriculture and Healthcare. 2014;4(21): 2224-8.
11. Engida, A., Novy, S.K., Yeshitila, A.T., Suryadi, I. et al. Extraction, identification and quantitative HPLC analysis of flavonoids from sarang semut (Myrmecodia pendans). Industrial Crops and Products. 2013;41: 392-6.
12. Daniel. Isolasi senyawa fenolik pada fraksi metanol-air dari umbi tumbuhan sarang semut (Myrmecodia tuberose Jack). J Kimia Mulawarman. 2010;8:1-6.
13. Soeksmanto, M.A., Subroto, H., Wijaya, P., Simanjuntak. Anticancer activity test for extracts of sarangsemut plant (Myrmecodya pendans) to HeLa and MCM-B2 cells. Pakistan Journal of Biological Sciences. 2010;13: 148-51.
14. Simanjuntak, P., Fanny, Subroto, M.A. isolasi senyawa aktif dari ekstrak hipokotil sarang semut (MymercodiaPendans) sebagai penghambat xantinoksidase. J Ilmu Kefarmasian Indonesia. 2010;15: 201-5
15. El-Khattouti, A., Selimovic, D., Haikel, Hassan, M. Crosstalk between apoptosis and autophagy: molecular mechanisms and therapeutic strategies in cancer. Journal of Cell Death. 2013;6: 37-40.

16. Selimovic, D., Sprenger, A., Hannig, M, Haïkel, Y., Hassan, M. Apoptosis related protein-1 triggers melanoma cell death via interaction with membrane region of p 75 neurotrophin receptor. J Cell Mol Med. 2012;16(2): 349–1.
17. Thomas et al. Upregulation of DR5 receptor by the diaminothiazole dat1 [4-amino-5-benzoyl-2-(4-methoxy phenyl amino) thiazole] triggers an independent extrinsic pathway of apoptosis in colon cancer cells with compromised pro and antiapoptotic proteins. Apoptosis. 2013;18(6): 713-26.
18. Cha, J.H. Allicin inhibits cell growth and induces apoptosis in u87mg human glioblastoma cells through an erk-dependent pathway. Oncol Rep. 2012;41-8.
19. Herzog, N., Hartkamp, J., Verheugd, P. Caspase-dependent cleavage of the mono-adp-ribosyltransferase artd 10 interferes with its pro-apoptotic function. FEBS J. 2013;280(5): 1330–43.
20. Rad, S.K., Kanthimathi, M.S., Malek, S.N.A., Lee, G.S., Looi, C.Y., Wong, W.F. Cinnamomum cassia suppresses caspase-9 through stimulation of akt1 in mcf-7 cells but not in mda-mb-231 cells. Plos One Journal. 2015;1-22.
21. Siti, N., Yanti, L., Apoptosis dan respon biologik sel sebagai faktor prognosa radioterapi kanker. Journal Buletin Alara. 2006;3(7): 25-7.
22. King, R.J.B.., Robins, M.W. Cancer biology. 3rd Ed. Pearson Prentice Hall. Harlow, England. 2006;153-60.
23. Supriatno. Cis-platinum meningkatkan apoptosis dan hambatan invasi sel kanker lidah manusia: in vitro. MIKGI. 2008;10: 73-8.
24. Hoeben, A., Landy, B., Highley, M., Wildiers, H., Ooseterom, A.T.V., Bruijn, E.D. Vascular endothelial growth factor and angiogenesis. Pharmacol Rev. 2004;56: 549-80.
25. Hicklin, D.J., Ellis, M. Role of vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol. 2005;23(5): 1011-27.
26. Story, M., Kodym, R. Signal transduction during apoptosis: implication for cancer theraphy. Frontiers in Bioscience. 1-5.
27. Hasanuddin, Krisnadi, R.S., Gandamihardja, S., Kurnia, D., Adhita, H.D. 2015 Terpenoid bioactive compound isolated from papua ant nest induces the apoptosis of human ovarian cell lines (skov-3) and increasing caspase-9 activity. American Journal of Research Communication. 1998;3(9): 1-8.
28. Nguyen, K.C., Willmore, W.G., Tayabali, A.F. Cadmium telluride quantum dots cause oxidative stress leading to extrinsic and intrinsic apoptosis in hepatocellular carcinoma hep g2 cells. Toxicology. 2013;306: 114–23.
29. Sulma, M. Commited to ensure quality publication in cancer research.. Clinmed International Library: USA. International Journal of Cancer and Clinical Research. 2014;1:1-2.
30. Wen-fu, T., Li-ping, L., Mei-hong, L., Yi-Xiang, Z., Yun-guang, T., Dong, X., Jian, D. Quercetin, a dietary-derived flavonoid, possesses antiangiogenic potential. European Journal of Pharmacology. 2002;459: 255-62.
31. Masferrer, J.L., Leahy, K.M. Antiangiogenic and Antitumor Activities of Cyclooxygenase- 2 Inhibitors. Cancer Res. 2000;60: 1306-11.
32. Chaudhary M, Bohra S, Gupta R, Patil S. Comparison and prediction of the extent of lesion of oral squamous cell carcinoma. Journal of International Dental and Medical Research. 2012; 5(2): 77-84.
33. Purwaningsih NMS, Sailan A, Sinon AHM, Jalil AA. Role of p16 and p53 in oral potentially malignant disorders and oral squamous cell carcinoma: a study in malaysia. Journal of International Dental and Medical Research. 2017; 10 (1): 42-47.
34. Purwaningsih NMS, Sailan AT, Jalil AA, Sinon SHM. Human papillomavirus detection in oral potentially malignant disorders and oral squamous cell carcinoma. Journal of International Dental and Medical Research. 2017; 10 (2): 198-201.