Evaluation of Anti-angiogenic Activity of *Centella asiatica* Water Extract on Chick Embryo Chorioallantoic Membrane Induced by bFGF

Iwan Sahrial Hamid* and Ngakan Made Rai Widjaja

Departement of Basic Veterinary, Faculty of Veterinary, Universitas Airlangga

Abstract

Angiogenesis is a physiological process that involves the formation of new blood vessels. In the cancer growth process, angiogenesis acts by supplying nutrition and oxygen for the cancer cells, carries metabolic waste and biological end product of cancer cells. Therefore the spread of cancer cells to the surrounding cells and tissues is accelerated. The occurrence of angiogenesis shows a change in cancer cell status, from dormant to malignant. Cancer cells induce the formation of angiogenesis through the secretion of various growth factors such as Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor (bFGF). Currently many people turn into treatment using natural materials. One of the natural materials used for cancer therapy is pegagan (*Centella asiatica*). In this research 25 embryonated chicken eggs (ECE) were divided into five treatment groups. Treatment group I as the control received 60 ng bFGF, treatment group II received neither bFGF nor pegagan leaves (*Centella asiatica*) water extract, and treatment groups III-V received 60 ng bFGF and *C. asiatica* leaves water extract of 75, 90 and 110 µg. Nine days old ESE were inoculated with bFGF and extract at the same time and then were incubated at 38°C for three days to allow the formation of blood vessels in embryonated chicken egg chorioallantoic membrane. Observations were performed microscopically and macroscopically by counting the new blood vessels formed around paper disc. Analysis on VEGF expression were conducted to define the role of angiogenesis growth factor. Research results showed a significant difference of treatment with *Centella asiatica* water extract on the number of new blood vessels formed. *Centella asiatica* water extract at the dose of 110 µg was the highest in inhibiting the formation of new blood vessels. *Centella asiatica* water extract also inhibited the expression of VEGF.

Keywords : *Centella asiatica*, antiangiogenic, Corio Allantois Membran, VEGF

INTRODUCTION

Cancer becomes health problem worldwide, since the accelerating morbidity and mortality caused by disease progression. According to *World Health Organization* (WHO), each year 6.25 millions of people are afflicted with cancer. It is predicted that 9 millions of people would die of cancer for the following 10 years (Malcolm, *et al.*, 2008). Cancer growth denoted carcinogenesis, begin with the development of one cell into small colony resided in the same tissue. This small colony undergoes genetic changes which leads to abnormality then malignancy (Bernard, 2000).

Angiogenesis is a physiological process that involves the formation of new blood vessels. Angiogenesis acts by supplying nutrition and oxygen for the cancer cells, carries metabolic waste and biological end product of cancer cells. Therefore the spread of cancer cells to the surrounding cells and tissues is accelerated. The occurrence of angiogenesis shows a change in cancer cell status, from dormant to malignant. Cancer cells induce the angiogenesis through the secretion of various growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Baker, 2007).

*Corresponding author email: kelana_dawley@yahoo.com

Basically, cancer treatment using chemotherapy or herbal medicines are not much different. Both treatments are effective to kill intracellular and extracellular cancer, which spread along the blood stream. Hitherto, cancer treatment by directly killing cancer cells is considered to be less effective because carcinogenic substituents of cells are still able to spread freely to other tissues *en route* blood vessels. Therefore, cancer treatment by inhibiting angiogenesis is more effective in treating the disease than to kill the cancer cells directly. Via angiogenesis inhibition, the supply of nutrients and oxygen to cancer cells will be hampered thoroughly, thus resulted in growth inhibition of cancer (Rafii, 2002). Current trend presented societal preference toward natural ingredients. One of the natural ingredient that provides novelty for cancer treatment is pegagan (*Centella asiatica*). Previous studies of *Centella asiatica* with asiatic acid as the active compound recommended the application of this plant in the prevention and treatment of cancer (Ruan, 2006; Zheng, 2007). Angiogenesis is contributed to cancer carcinogenesis in the early development of disease. Then, this is the aim of this research to evaluate the role of *Centella asiatica* to influence angiogenesis.

MATERIALS AND METHOD

Sample Preparation

Fresh leaves of *Centella asiatica* were washed thoroughly with flow water, drained, and dried by exposing indirectly to the sun heat (covered with dark cloth). The sun-dried leaves were then blended and shieved into fine powder. As many as 5 g of powders was put into a glass beaker, then added distilled water with a temperature of 28°C to reach a volume of 200 mL. This process was carried out in an incubator for 24 hours. After that, the mixture was filtered with Whatmann paper no. 4 in order to get the water extract. The extract was then dried with a freeze dryer and stored in a refrigerator with a temperature of 10°C.

Chemical

Water extract of *Centella asiatica* was dissolved in aquadest for test purpose. Angiogenic inductor used in this research was human recombinant bFGF 10ng/μL. Nine days old of embryonated chicken eggs (ECE)

specific pathogenic free were used in incubated condition. The 0.01 M Tris-HCl pH 7.5 was used as bFGF solvent. 10 % phosphate buffered formalin used to preserve CAM membranes.

Anti-angiogenic Assay

Anti-angiogenic assay was performed on 9 days old ECE chorioallantoic membrane (CAM). A hole was drilled gently on the surface of ECE shell to form a rectangular area with 1 cm² in width. Through this hole the test solution was implanted using media paper disc (Ribbati, *et al.*, 2007). ECE were divided into 7 groups, each treatment was consisted of five ECE. Treatment I: positive control, 60 ng bFGF administration. Treatment II: negative control, solvent (Tris-HCl and aquadest) administration. Treatment III: 60 ng bFGF and 75 μg water extract of *Centella asiatica* in aquadest. Treatment IV: 60 ng bFGF and 90 μg water extract of *Centella asiatica* in aquadest. Treatment V: 60 ng bFGF and 110 μg water extract of *Centella asiatica* in aquadest. Treatment VI: positive control group of synthetic antiangiogenesis drug 20 μg Celecoxib and 60 ng bFGF administrations. Treatment VII: negative group, 110 μg water extract of *Centella asiatica* in aquadest. After administrated with each treatment, ECEs were incubated in 38–39°C with 60% relative humidity for 72 hours. The ECE were then opened, removed egg contents gently to keep CAMs remain intact to the eggshell. New blood vessels around the paper discs were counted. To observe the number of VEGF expression, immunohistochemistry was performed. The CAMs around paper discs were collected in 10% buffered formalin for HE staining.

Data Analysis

The number of microscopic and macroscopic new blood vessels formed during observation were analyze using parametric analysis of variance F-test. To compare the differences of means among each treatment group, Duncan multiple comparison test with 95% confidence interval was employed. Using statistical software SPSSTM version 16.0 descriptive analysis of VEGF expression was conducted by comparing the results of imunohistochemical staining techniques from each treatment group.

RESULTS AND DISCUSSION

The results of macroscopic observation and comparison between bFGF control group untreated with extract and group treated with extract were shown in Fig.1. Observations were followed by counting the number of new blood vessels or capillaries that extend and branch out from great vascular origin. The results of the calculation were presented as means of four replications from each treatment group. The means values of the number of new blood vessels can be seen in Table I. There was no significant difference in the number of blood vessels reduction for each concentration. The difference of means for each treatment was analyzed using Duncan multiple range test. The lowest means value of the number of blood vessels was 110 µg of *Centella asiatica* water extract + 60 ng bFGF treatment group, with the value of $2.60^c \pm 1.14$. These results were not different significantly ($p > 0.05$) with Tris solvents and DMSO control group, with the

value of 3.20 ± 0.83 . It indicates that 110 µg of *Centella asiatica* water extract has equal activity with solvent control group to inhibit the development of new blood vessels induced by 60 ng bFGF. The 75 µg and 90 µg of *Centella asiatica* water extract treatment group were not different significantly. Nevertheless, the treatment groups showed inhibition activity toward new blood vessels development ($4.00^{bc} \pm 0.70$; $4.40^b \pm 1.51$; $2.60^c \pm 1.14$) compared to 60 ng bFGF control group ($7.00^a \pm 1.22$). The 20 µg celecoxib positive control group gave means value as much as $2.80^c \pm 0.84$, it was not different significantly compared to 110 µg of *Centella asiatica* water extract treatment group.

The administration of *Centella asiatica* water extract for each group showed dose dependent manner. These results indicate that the higher concentration of *Centella asiatica* water extract will resulted in lower development of new blood vessels. The percentage of new blood vessels reduction for each treatment group is listed in Table 2.

Figure 1. The development of blood vessels around paper disc that was placed directly on the CAM. Two images above are bFGF control group untreated with extract. Lower left and lower right images are the group treated with 90 µg of extract and 110 µg of extract, respectively.

Table 1. The means values of the number of new blood vessels on CAM from each treatment group based on macroscopic observation

Treatment groups	The number of new blood vessels (Means $\pm$ SD)
60 ng bFGF control	7.00 ^a $\pm$ 1.22
Tris and DMSO solvent control	3.20 ^{bc} $\pm$ 0.83
Treatment II : 75 μ g of <i>Centella asiatica</i> extract + 60 ng bFGF	4.40 ^b $\pm$ 1.51
Treatment III : 90 μ g of <i>Centella asiatica</i> extract + 60 ng bFGF	4.00 ^{bc} $\pm$ 0.70
Treatment IV : 110 μ g of <i>Centella asiatica</i> extract + 60 ng bFGF	2.60 ^c $\pm$ 1.14
Positive control : 60 ng bFGF + 20 μ g Celecoxib	2.80 ^c $\pm$ 0.84
Negative control : 110 μ g <i>Centella asiatica</i> extract	3.40 ^{bc} $\pm$ 0.54

Table 2 . The percentage of new blood vessels reduction in CAM compared to the bFGF control group

Treatment groups	Percentage of blood vessels reduction (%)
60 ng bFGF control	---
Tris and DMSO solvent control	54.28
Treatment II : 75 μ g of <i>Centella asiatica</i> extract + 60 ng bFGF	37.14
Treatment III : 90 μ g of <i>Centella asiatica</i> extract + 60 ng bFGF	42.85
Treatment IV : 110 μ g of <i>Centella asiatica</i> extract + 60 ng bFGF	62.85
Positive control : 60 ng bFGF + 20 μ g Celecoxib	60.00
Negative control : 110 μ g <i>Centella asiatica</i> extract	51.30

The highest value in the percentage of new blood vessels reduction was 110 μ g of *Centella asiatica* water extract + 60 ng bFGF treatment group, with the value of 62.85%. This was not different significantly ($p>0.05$) compared to Tris-HCl and DMSO solvent control group (54.28%). Whereas 75 μ g and 90 μ g of *Centella asiatica* water extract treatment group showed percentage of new blood vessels reduction, with the value of 37.14% and 42.85%, respectively. Both treatments were not different significantly. Almost all treatment groups achieved percentage of new blood

vessels reduction in average above 40%, indicating the success of *Centella asiatica* leaf extracts to inhibit the development of new blood vessels (angiogenesis).

Microscopic observation toward blood vessels formation on CAM is necessary in order to validate macroscopic observation. This observation was carried out by calculating the formation of new blood vessels around the major veins. Conducted on three point of views, the means values were then calculated, results can be seen in Table 3.

Table 3. The means values of the number of new blood vessels on CAM from each treatment group based on microscopic observation

Treatment groups	The number of new blood vessels (Means $\pm$ SD)
60 ng bFGF control	93.77 ^a $\pm$ 6.51
Tris and DMSO solvent control	36.28 ^c $\pm$ 2.71
Treatment II : 75 μ g of <i>Centella asiatica</i> extract + 60 ng bFGF	55.72 ^b $\pm$ 5.72
Treatment III : 90 μ g of <i>Centella asiatica</i> extract + 60 ng bFGF	48.62 ^b $\pm$ 8.95
Treatment IV : 110 μ g of <i>Centella asiatica</i> extract + 60 ng bFGF	51.06 ^b $\pm$ 11.59
Positive control : 60 ng bFGF + 20 μ g Celecoxib	39.20 ^c $\pm$ 2.38
Negative control : 110 μ g <i>Centella asiatica</i> extract	36.60 ^c $\pm$ 5.03

Microscopic calculation of new blood vessels formation on CAM showed significant difference on means value for each *Centella asiatica* extract treatment group (75, 90, and 110 µg), but not highly significant ($p < 0.01$). Compared to solvent control group, there is no significant difference on means value of *Centella asiatica* extract treatment group ($p > 0.05$). Nevertheless, the results showed that *Centella asiatica* water extract successfully inhibit the formation of new blood vessels induced by 60 ng bFGF. Histopathologic image of new blood vessel formation showed cross-sectional capillaries formed amidst CAM. Result can be seen in Fig. 2.

Microscopically, new blood vessels denoted as blood vessels that grow and evolve from main veins. They are characterized by round to oval lumens, vascular walls resemblance to thin membranes, homogenous, unaccompanied by another muscles, and have rarely visible endothelial nuclei and blood cells in its lumen. The formation of

new blood vessels presented in Fig. 2 were shown on the tip of the black arrow. Reduction in capillary blood vessels density was observed on each treatment. Newly formed blood vessels begin to decrease progressively by increasing concentration of *Centella asiatica* water extract.

VEGF expression is present on endothelial cells of new blood vessels, which branch out from main veins. This new blood vessels usually appear around the main veins. VEGF expression in cytoplasm or on the surface of endothelial cells can be detected using anti-VEGF antibody. The administration of 60 ng bFGF in treatment group resulted in VEGF expressions which were dominated by the presence of brown color. The reduction in brown color which indicate a lower expression of VEGF was shown after the administration of *Centella asiatica* water extract. This phenomenon was appear in dose dependent manner. The result from microscopic observation of VEGF expression is presented in Fig. 3.

Figure 2. Cross-sectional capillaries formation on CAM for each treatment group, microscope with 400x magnification. (A) bFGF positive control, (B) solvent control, (C) 75 µg extract + bFGF, (D) 90 µg extract + bFGF, (E) 110 µg extract + bFGF. The arrow (—→) showed the formation of new blood vessels.

Figure 3. VEGF expressions in the treatment group are indicated by black arrows. (A) bFGF positive control without *Centella asiatica* extract, (B) solvent negative control, (C) 75 µg of *Centella asiatica* water extract treatment group, and (D) 110 µg of *Centella asiatica* water extract treatment group.

Active compounds in *Centella asiatica* water extract are responsible in its anti-angiogenesis activity. These active compounds comprising triterpenoids, for instances aglycone asiatic acid, madecassoside, and madasiatic acid. The vascular effect of triterpenoids is related to inhibition of collagen production. Collagen has an important role in the wound healing process by improving vascular endothelial (Gohil, *et al.*, 2010). Among these triterpenoids, aglycon asiatic acid is dominant to inhibit the expression of angiogenic factor VEGF. It is in accordance with the research conducted by Kavitha, *et al.* (2011) that 5-20 µM of asiatic acid inhibits stimulation factor for VEGF expression and formation of capillary tubes. Based on this study, it is concluded that *Centella asiatica* water extract inhibits the growth of new blood vessels on CAM induced by bFGF as the result of microscopic and macroscopic observations, and can inhibit the

expression of VEGF. As many as 110 µg of *Centella asiatica* water extract provide highest barrier for the formation of new blood vessels.

REFERENCES

- Baker, T., 2007, *Enzyme Eliminated by Cancer Cells Holds Promise for Cancer Treatment*, Website, http://www.eurekalert.org/pub_release_s/2007-07/mcog-eeb071807.php.
- Gohil, K.J., Patel, J.A. and Gajjar, A.K., 2010, Pharmacological review on *Centella asiatica*. A Potential Herbal Cure-all, *Indian J. Pharm. Sci.*, **72**(5), 546-556.
- Kavitha, C.V., Agarwal, C., Agarwal, C.R. and Deep, G., 2011, Asiatic Acid Inhibits Pro-angiogenic Effects of VEGF and Human Gliomas in Endothelial Cell Culture Models, *Plos One.*, **6**(8), 22745.

- Moore, M.A., Manan, A.A., Chow, K.Y., Cornain, S.F., Devi, C.R., Triningsih, F.X., *et al.*, 2008, Cancer Epidemiology and Control in Peninsular and Island South-East Asia - Past, Present and Future, *Asian Pac. J. Cancer Prev.*, **11**(suppl 2), 81-98.
- Rafii, S., Heissig, B. and Hattori, K., 2002, Efficient Mobilization and Recruitment of Marrow-Derived Endothelial and Hematopoietic Stem Cells by Adenoviral Vectors Expressing Angiogenic Factors, *Gene Ther.*, **9**(10), 631-641.
- Ruan, W.J., Lai, M.D. and Zhou, J.G., 2006, Anticancer Effects of Chinese Herbal Medicine, Science or Myth?, *J. Zhejiang Univ. Sci. B.*, **7**, 1006-1014.
- Weinstein, I.B., 2000, Disorders in Cell Circuitry During Multistage Carcinogenesis: The Role of Homeostasis, *Carcinogenesis*, **21**(2), 857-864.
- Zheng, C.J. and L.P. Qin., 2007, Chemical Component of Centella asiatica and their Bioactivities, *Zhong Xi Yi Jie He Xue Bao.*, **5**(3), 348.351.