ANTIANGIOGENIC EFFECTS OF WATER FRACTION OF GREEN ALGAE (*Spirogyra* sp.) ETHANOL EXTRACT WITH CHORIO ALLANTOIC MEMBRANE METHOD

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Abstract

Background. *Angiogenesis plays an important role in tumor progression. Therapeutic angiogenesis is a major focus in cancer treatment. Modern treatment of cancer currently has alternative to inhibit cancer through antiangiogenesis process, it is expected to inhibit the formation of new blood vessels around the tumor, the supply of nutrients and oxygen by the blood to the tumor can be inhibited. This study aims to determine the fraction of water* ***antiangiogenesis effect*** *of ethanol extract of green algae (Spirogyra sp.) by using the method of chorio allantoic membrane (CAM).*

Methods. *In this study subject were devided into 7 groups; group I of the control disc, group II on the disc+PBS control group, group III of the control disc+PBS +bFGF, variation in the concentration of the water fraction of ethanol extract of green algae (Spirogyra sp.) is group IV, V, VI, VII which is each of these group has variation of doses 100, 200, 400 and 800 µg/ml. CAM is obtained from embryonated chicken eggs aged 8-9 days, then it is given treatment bFGF (angiogenesis inductor) and water fractions of ethanol extract of green algae (Spirogyra sp.), and then incubated for 3 days.*

Results and Discussion. *Observations are made in the macroscopic to see the inhibitory activity of blood vessels from the water fraction of ethanol extract of green algae (Spirogyra sp.). Having obtained the result of macroscopic observation, the data of the number of blood vessels were tested statistically by the method of Mann Whitney and Kruskal Wallis with 95% confidence level.*

Conclusions. *The results showed that the water fraction of ethanol extract of green algae with doses 100, 200, 400 and 800 µg/ml can inhibit angiogenesis of embryonated chicken eggs compared to bFGF control. In conclusion, the water fraction of ethanol extract of green algae has antiangiogenesis activity in chorio allantoic membrane.*

Keywords . *antiangiogenesis, CAM, green algae*

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INTRODUCTION

Cancer can grow in any cell or tissue, such as skin cells, blood cells, brain cells, lung cells, liver cells, connective tissue and many more. Therefore, the known various types of cancer, depend on the growth of cells or tissues. Each type of cancer has a growth rate of reactions on different treatments (Dalimartha, 2004). To be able to grow and develop, the cancer requires a series of process called carcinogenesis that has the features such as increased mobility and angiogenesis (Schneider, 1997).

Angiogenesis is the process of forming new blood vessels in the body or neurovascularisation derived from the old blood vessels. Angiogenesis is controlled by certain chemicals produced in the body. Some of these chemicals can stimulate cells to repair the damaged blood vessels or the form of new blood vessels. Other chemicals is called angiogenesis inhibitors, give a signal to the cell to stop the process of angiogenesis. For the process of angiogenesis, among the other of necessary vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) is an angiogenic peptide that is potentially on the development of hematopoietic stem cell control and modification of extracellular matrix (Anonimc, 2011).

Quantitative methods and angiogenesis inhibition continue to be developed and have been used to investigate, both in vivo and in vitro (Ribatti et al, 1997). Observations in response to the tumor angiogenesis in vivo implantation of tumor tissue can perform on the organs that contain lots of blood vessels. Organ are often used in the implantation of tumor tissue such as rabbit cornea, skin on the back of mice and also it can be used chorio allantoic chick embryo membrane (CAM) for the in situ observations of angiogenesis (Ribatti et al, 1997).

Green algae is a plant that containing the active substance in the form of phytomeatonin. Phytomelatonin is a melatonin that found in plants. Phytomelatonin levels of compounds in green algae is 240 µg/kg wet weight (Kolar and Machackova, 2001).

Meltonin is an alkaloid compounds act as inhibitors and anti cancer activity (Veronique et al, 2005). Melatonin works with three actions in the inhibition of cancer activity, which include working with specific cytotoxic properties, slowing the division (proliferation) of cancer cells, and negate the toxic effects of heavy metals (epigenetic carcinogens). Usefulness of this compound is known as cancer activity by inhibiting the cytotoxicity test and anti proliferative test.

With the reference above the explanation it is necessary to do research on anti angiogenesis effects of green algae plants using the method of chorio allantoic membrane (CAM).

METHOD

Material

Materials use in this study is the water fraction of green algae (Spirogyra sp.) ethanol extract. Obtained by isolation of green algae in the laboratory of plant pharmacognotion and phytochemistry Faculty of Pharmacy, Ahmad Dahlan University Yogyakarta, chorio allantoic membrane (CAM) that derived from the chicken egg specific pathogen free (SPF), bFGFm antiseptic solution, sterile distilled water.

Extraction

Processing of green algae to a powder requires several stages. The stages are: 1) green algae cleaned from impurities by washing with the water flow. 2) algae put in a container of water and allowed to stand for one day (12 hours with radiation and 12 hours with irradiation phase of the phase fraud. 3) algae taken from the container at 5 hours after the phase of embezzlement and cut into pieces with a thickness of 1-5 mm. 4) the pieces are dried in the sun to dry under the sun is covered by black cloth. Closure of the black cloth is intended to speed up the drying process as a black cloth can absorb heat and also to avoid damage due to the

active substance from the sun's UV rays. 5) algae is pulverized then dried using a blander. the pulverizing process can increase the surface area between the simplicia solution. This process can increase the number of active substances in the summarized extract. Green algae that has been in the form of powder extraction with several stages, namely: 1) green algae powder was extracted with 96% ethanol bu Soxhlet method. 2) the summary of green algae ethanol extract then evaporated using a rotary evaporators with the temperature is 40º C to obtain a thick extract. 3) the results of the condensed extract is then performed by using a solvent-water fractionation, fractionation carried out until the color of water becomes colorless again, which means the water-soluble fraction was summarized into the water solvent.

Test Inhibition of Angiogenesis

Embryonated chichen eggs used on 8-9 days old. bFGF and extract are implanted into the membrane of chorio allantoic through holes in the air space that has been moved above the position of the embryo. Eggs were divided into seven groups (each treatment group consisted of five eggs). Group I was an egg with a paper disc implantation, as a control of paper disc. Group II is the implantation of the paper disc with PBS as a solvent control group. Group III, with implantation of 10 µg bFGF+ 20 µl PBS+paper disc, as a control of bFGF. Group IV, V, VI, VII are eggs that are used to see the inhibitory effect of water fraction of green algae ethanol extract on CAM angiogenesis. Eggs in this group were the implantation of bFGF 10 µg with paper disc and water fraction of green algae ethanol extract with doses 100, 200, 400 and 800 µg/ml. Once treated, then the eggs were incubated at 39º C and 60% relative humidity for 3 days or 72 hours (Ribatti et al, 1997). On the three days after incubation eggs (age 12 days) was opened and the membrane chorio allantoic attached to the shells observed macroscopically.

Analysis of Results

Anti angiogenesis test evaluation is done by observing the macroscopic response of hospes angiogenesis (blood vessel response CAM) is descriptive and quantified by counting the number of new vessels on disc or paper around the paper disc (radial pattern), then using a modified method that is scoring method Knighton *et al,* (1997). To reduce the subjectivity of the observations, the observations made by three people, so the result is an average of three such observations. Observations of new blood vessels that from on and around the paper disc, must be distinguished from the main blood vessels/origin of the CAM. Where the main blood vessels/CAM has a home at a larger size, while the new blood vessels are arteries more subtle/minor (Ribatti *et al*, 1999).

The results of quantifications of the amount of new blood vessels can be calculate using the formula of % inhibitory:

$$\%\text{inhibition} = \frac{\sum \text{blood vessels bFGF control} - \sum \text{treatment blood vessels}}{\sum \text{blood vessels bFGF control}} \times 100\%$$

The results of quantification of the amount of new blood vessels and then statistically analyzed using the Mann Whitney and Kruskal Wallis ($p = 0{,}05$).

RESULT AND DISCUSSION

Indication of CAM angiogenesis

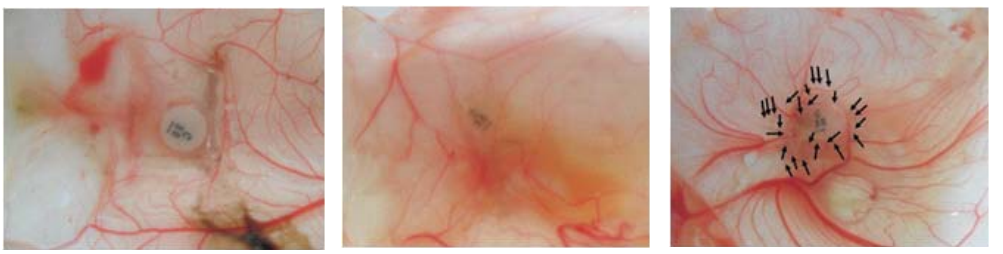
The method used to determine whether the fraction of water from the ethanol extract of green algae have anti cancer effects in through inhibition of angiogenesis using the chorio allantoic membrane (CAM) that have been induced by basic fibroblast growth factors (bFGF). CAM method has several advantages, namely embryonated chicken eggs readily available, relatively inexpensive, easy to work in

the laboratory and more practical. Another plus is in embryonated chicken eggs to create a closed environment and protected by a shell of the egg, making it safe, easy to hold and maintained during the incubation in the laboratory. Closed environment is relatively constant because of the existence of extra-embryonic fluid and some wrapping membrane in embryonated chicken eggs (Evans, 1991).

CAM chicken embryo it self in one of the most common media used to study the response of angiogenesis. This is because the CAM is a membrane that is rish in blood vessels (Ribatti *et al,* 1997). So the observation of the angiogenesis response is easier to observe. Eggs are used in this study was ten days, this is because at the age of ten days on the CAM blood vessels are more stable. While the tools used to test the previous anti angiogenesis sterilized to avoid contamination by bacteria or microbes that a scan affect process of testing anti angiogenesis. Water fraction of green algae ethanol extract was dissolved in a PBS solvent, PBS was chosen because it can dissolve the water fraction of green algae ethanol extract are also safe to use on eggs used un anti angiogenesis trials.

As a trigger angiogenesis is embryonated chicken eggs membrane (CAM) is used bFGF. Giving bFGF to induce angiogenesis is the CAM made as occurs in tumor tissue exposed. With the introduction of bFGF, the inhibitory effect of angiogenesis on CAM by the water fraction of green algae ethanol extract can be observed more clearly. In this observation of macroscopic visible small blood vessels around the paper disc, sometimes it is a ramification of the vascular origin. Positive control used were bFGF and PBS soven, where as the negative control used was paper disc only. The use of paper disc as a negative control due to limitations in getting the anti angiogenesis drug that has been standardized by the method of CAM so that negative control is a control in the absence of bFGF implantation.

Observations in the control group paper disc + bFGF + PBS showed an increase in the number if new blood vessels are aplenty in the area around the paper disc, so it can be said to indicate the presence of angiogenesis effects. New blood vessels are formed with a radial pattern to the paper towards the disc, it is clear that administration of bFGF actually induce angiogenesis in the CAM. While the paper disc in the control group + PBS showed similar result with a control group who were given only by the paper disc only, no increase of the new form of new blood vessels such as paper disc in the control group PBS + bFGF. The third appearance of the control group differences in macroscopic or visual can be seen in figure 1.



(1). Paper disc (2). PBS solvent (3). bFGF

Figure 1. Macroscopic observation on the group control

Information : (1). paper disc control (2). paper disc + PBS control, (3). paper disc + PBS + bFGF control.

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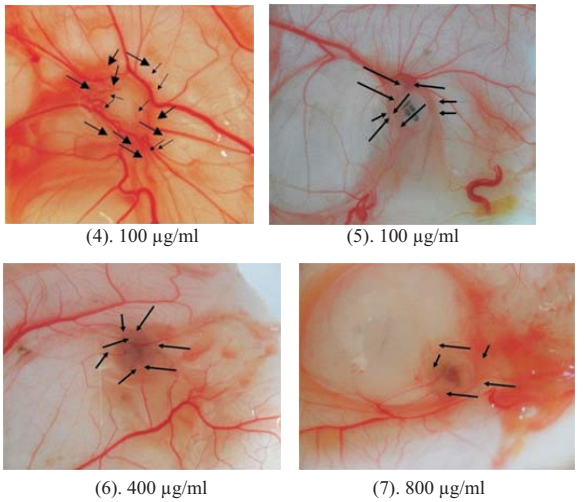

(4). 100 µg/ml (5). 100 µg/ml

(6). 400 µg/ml (7). 800 µg/ml

Figure 2. Macroscopic observation in CAM were induced bFGF in treatment group.

information : (4). Water fraction with doses 100 µg/ml, (5). Water fraction with doses 200 µg/ml, (6). Water fraction with doses 400 µg/ml, (7). Water fraction with doses 800 µg/ml, (⟶). New blood vessels

Effect of water fraction of green algae on bFGF- induced CAM

Macroscopic observation can indicate that the water fraction of green algae ethanol extract could inhibit new blood vessel growth or angiogenesis in the CAM induced by bFGF.

Angiogenesis assay results of water fraction of green algae ethanol extract showed the higher level, the higher the inhibition of angiogenesis that occurs in bFGF-induced CAM is characterized by a decrease in the density score of new blood vessels. In the egg with a concentration of 100 µg / ml are seen a lot of new blood vessel growth in the area around the paper disc. New blood vessel growth is experiencing a reduction in the concentration of 200, 400, 800 µg/ml. However, the optimum concentration that can provide anti angiogenesis effect of ethanol extract of the water fraction of green algae could not be determined because of the difficulty of quantification of new blood vessels on the CAM. Therefore we need the other methods to determine how much water fraction is the ability to inhibit angiogenesis. Test group differences in the appearance of the water fraction of ethanol extract are shown in Figure 2.

Table I. Quatification of new blood vessels in a control and treatment group beside on macroscopic observation

PBS solvent	Number of new blood vess el growth
Paper disc	0 ± 0
PBS solvent	0 ± 0
bFGF control	26 ± 0
water fraction of green algae ethanol extract 100 µg/ml	12,25 ± 3,5
water fraction of green algae ethanol extract 200 µg/ml	9,25 ± 0,5
water fraction of green algae ethanol extract 400 µg/ml	7 ± 0
water fraction of green algae ethanol extract 800 µg/ml	4,67 ± 0,57

Presentation % inhibition could also be obtained by comparing the number of blood vessels in the treated group by the number of new blood vessels in the control group bFGF. It is

useful to know what % inhibition of the water fraction of ethanol extract of green algae on angiogenesis. Procentation % inhibition can be seen in Table II:

and homogeneous or inhomogeneous. Based on preliminary test results in the test group, indicating that the data were normally distributed, but not homogeneous so that the

Table II. The price of % inhabition of water fraction of green algae ethanol extract

Treatment	% inhibition
bFGF control	0 ± 0 %
water fraction of green algae ethanol extract 100 µg/ml	52,88 ± 13,46 %
water fraction of green algae ethanol extract 200 µg/ml	64,41 ± 1,925 %
water fraction of green algae ethanol extract 400 µg/ml	73,07 ± 0 %
water fraction of green algae ethanol extract 800 µg/ml	82,04 ± 2,23 %

Data on the number of new blood vessels group in each treatment were then analyzed statistically. Statistical analysis was performed to determine the difference of each test group and to further reinforce the conclusion of the treatment decision. The first step in a statistical test is to determine whether the test group was normally distributed or not normally distributed analysis performed is non-parametric analysis by using the Mann Whitney test and Kruskal-Wallis test. From the results obtained it can be concluded that there were significant differences between treatments. To find treatment anywhere that shows significant differences used Mann-Whitney. More results shown on Table III.

Table III. Resume of analysis statistic with Mann Whitney test in control and treatment group

Variation	paper disc control	PBS control	bFGF control	Water fraction 100 µg/ml	Water fraction 200 µg/ml	Water fraction 400 µg/ml
PBS control	TS (p=1,000)					
bFGF control	S (p=0,025)	S (p=0,025)				
Water fraction 100 µg/ml	S (p=0,037)	S (p=0,037)	S (p=0,037)			
Water fraction 200 µg/ml	S (p=0,034)	S (p=0,034)	S (p=0,034)	S (p=0,046)		
Water fraction 400 µg/ml	S (p=0,025)	S (p=0,025)	S (p=0,025)	S (p=0,037)	S (p=0,034)	
Water fraction 800 µg/ml	S (p=0,034)	S (p=0,034)	S (p=0,034)	S (p=0,046)	S (p=0,043)	S (p=0,034)

Keterangan : S = different significant

TS = different not significant

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In this study, the provision of water fraction of ethanol extract of green algae proved capable of inhibiting the formation of new blood vessels. Inhibition is possible because of the compounds contained in the water fraction of ethanol extract of green algae that have activity as inhibitors of angiogenesis.

Angiogenesis is investigated in this study angiogenesis of cancer but not normal angiogenesis. Normal angiogenesis may be analogous to the mechanism of angiogenesis in cancer, because cancer also express a growth factor such as bFGF.

Angiogenesis is the formation of new blood vessels branch into cancer cells that will supply nutrients and oxygen needs of the cancer cells. Angiogenesis allows cells to receive nutrients and oxygen supply so that it can continue to survive. Some cells produc growth factors that can induce angiogenesis such as basic fibroblast growth factor (bFGF) and Vascular Endothelial Growth Factor (VEGF). So that the cancer cells will proliferate into a large period (Hanahan and Weinberg, 2000). Mechanisms that occur in the release of bFGF as a growth factor, namely bFGF interacts with endothelial cells through a receptor tyrosine kinase receptors and heparin sulfate proteoglycan (HSPGs) on the cell surface. Balance between storage and release of bFGF in the extra cellular matrix is an arrangement may be biological effects of these growth factors in the endothelium (Sugiyanto, 2009). So for the inhibition of angiogenesis, the neutralization of bFGF is sufficient to disrupt the balance and lead to angiogenesis inhibitation.

Based on this research, it is known that the water fraction of ethanol extract of green algae capable of inhibiting angiogenesis although not yet certain about the mechanism of inhibitory action. The possible mechanism of inhibition can be through various means, namely by inhibiting cell proliferation through growth factor endothelia, inhibits cell invasion by anti integrin inhibitor, prevents endothelia cell invasion in cancer cells by inhibitors of MMPs (metalloproteinases) and by increasing the concentration of endogenous inhibitors such as endostatin (King,2000). Inhibition of angiogenesis can be done through some goal or target. First, the targetaction and antiangiogenesis is a function of endothelia cell migration, inhibition of the vascular endothelia cadherin (VE cadherin) in endothelia cells serves to make bonds homotipic, meaning that the extracellular region of the molecule may be a closer relationship (Bosman, 1999). In addition to cadherin, integrins are also involved in transmitting signals to stimulate cell proliferation. On angiogenesis integrin $\alpha 5\beta 1$ integrins involved dalah and $\alpha v\beta 3$ (King, 2000).

Antibodies to VE cadherin and integrins can inhibit angiogenesis according to the study. Inhibitors of MMPs (metalloprotein) enzyme that plays a role in the degradation of extracellular matrix to facilitate cell entry in endothelia and spread of tumor cells that can function as a supplier of oxygen and nutrients to cancer cells (Kleinsmith *et al.* 1999). Target on angiogenic factor and its receptor (bFGF, VEGF, angiopoitins) with a humanized anti-VEGF / FGF monoclonal antibody is a potential therapy for solid tumors. Angiogenesis inhibitor type of antibody is known to inhibit cell proliferation and spread of vascular endothelia (Anonm, 2000) inhibitoe ofreceptors including the SU5416 and ZD4190, another example of the type-specific monoclonal antibody that is able to reduce the size of blood vessels in tumors is AA98.

Therapeutic angiogenesis at the molecular level caused by the induction of growth factor bFGF and involves complex mechanisms that couple. Inhibitory mechanism at the molecular level is the inhibition of the pathway tyrosine/threonine kinase pathway in SOS, Ras, Raf, and MAP kinase pathways. Ras is one of the main factors that signal transduction is activated through phosphorylation (a change in GNP, Ras becomes GTP-Ras) activation of Ras with GTP binding may lead to extracellular matrix adhesion and proliferation of cancer cells, in this case is endothelial cell activation during on going proliferation Ras (Ras GTP) will bind to

aprotein kinase. Raf, the cell membrane, Raf kinase through MAP kinase pathways (mitogen-activated protein kinase) would affect the activation of transcription factors in the cell nucleus to alter gene expression (Casanova *et al* 2002, Gibs, 2001).

Data on the number of new blood vessels of each treatment can also be calculated with % inhibitory prices and then statistically analyzed. Statistical analysis was performed to determine the difference of each test group and to further reinforce the conclusion of the treatment decision. Initial steps are performed in the same statistical test as the test statistic for the treatment of angiogenesis. From the results obtained it can be concluded that there were significant differences between the control group treated with bFGF. To find treatment anywhere that shows significant differences used Mann-Whitney. More results shown on Table IV.

but it is different when compared between the test compound water fraction of ethanol extract of green algae at a concentration of 100 and 200 µg/ml showed a different significance values are not meaningful, this is because the water fraction of the test compound ethanol extract of green algaeat a dose of 100 and 200 µg/ml showed no difference in concentration % inhibition of a fairly large when compared with other concentration of the test group. Can be concluded that the higher dose of test compound variations of water fractions of ethanol extract of green algae is a dose of 100, 200, 400 and 800 µg/ml, the higher the concentration % inhibitory means the test compound water fraction of ethanol extract of green algae has the ability to inhibit new blood vessel growth or angiogenesis.

Tabel IV. Resume of analysis statistic % inhibitory statistical analysis with Mann Whitney test in control and treatment group.

Variation	bFGF control	Water fraction 100 µg/ml	Water fraction 200 µg/ml	Water fraction 400 µg/ml
Water fraction 100 µg/ml	S (p=0,028)			
Water fraction 200 µg/ml	S (p=0,022)	TS (p=0,237)		
Water fraction 400 µg/ml	S (p=0,025)	S (p=0,028)	S (p=0,022)	
Water fraction 800 µg/ml	S (p=0,034)	S (p=0,032)	S (p=0,026)	S (p=0,034)

Keterangan : S = different significant
TS = different not significant

Based on the results of statistical analysis can be concluded that the price control % inhibition of bFGF in the group treated with the test compound is the water fraction of ethanol extract of green algae inhibitory effect in the formation of new blood vessels because of the significance values obtained by different means,

CONCLUSION

Water fraction of ethanol extract of green algae (Spirogyra sp.) has inhibitory activity of new blood vessel formation.

Water fraction of ethanol extract of green algae (Spirogyra sp.) capable for inhibiting the

formation of new blood vessels at concentrations of 100, 200, 400 and 800 µg/ ml.

Water fraction of ethanol extract of green algae contains phytomelatonin

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