INTERNATIONAL CONFERENCE ON CENTRAL MANAGEMENT OF CENTRAL CYTOTOXIC RECONSTITUTION

By HARI SUSANTI

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PROCEEDING

INTERNATIONAL CONFERENCE ON CENTRAL MANAGEMENT OF CENTRAL CYTOTOXIC RECONSTITUTION

Grand Cokro Hotel Yogyakarta
May 25th, 2013

THE INTERNATIONAL CONFERENCE ON CENTRAL MANAGEMENT OF CENTRAL CYTOTOXIC RECONSTITUTION IN PHARMACY PRACTICE YOGYAKARTA, INDONESIA, 2013

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Welcome Address from Chairman of Organizing Committtee

Honorable Rector of Ahmad Dahlan University,
Dean Faculty of Pharmacy of Ahmad Dahlan University,
Honorable Plenary Speakers
Dear Colleague,
Distinguished Participants,
Ladies and Gentlemen

Assalamu'alaikum warahmatullahi wabarakatuh,

Good morning,

First of all, let us give thanks to Allah, the Almighty God, who has allowed us to attend this conference. Secondly, I would like to welcome everyone to Yogyakarta.

Preparation and reconstitution of drug products are an essential function of hospital pharmacy. The hospital pharmacist should also take notice for central intravenous additive service including cytotoxic reconstitution services. Thus, handling cytostatic in hospital is also crusial in hospital practises. In other words, Cytotoxic Handling should to provide protection to patients, the operator and environment.

Ladies and gentlemen,

To celebrate the 17th aniversary faculty of Pharmacy Ahmad Dahlan University, Yogyakarta, in collaboarting with Bethesda Hospital, Yogyakarta, Indonesia hosted The International Conference on Safety Management of Central Cytotoxic Reconstitution in Pharmacy Practice". The conference is held on 25 May, 2013 at The Grand Tjokro Hotel, Yogyakarta, Indonesia. The conference facilitated some of professional that come from world wide such as academia, researchers, hospital pharmacists, policy maker, and health care professionals. The second agenda is Workshop on Basic Cytotoxic Handling for hospital pharmacists and academia. It will hold on 26-27 May, 2013 in Pharmacy Department, Bethesda Hospital.

I do hope this conference will give a new initiative for practicing sorounding cytotoxic handling and team-work. I thank to conference sponsors and committee members for their support.

Wassalamu'alaikum warahmatullahi wabarakatuh,

Yogyakarta, 25 May 2013

Dr.rer.nat. Endang Darmawan, M.Sc., Apotheker
Chairperson of the organizing committee

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ANTI ANGIONESIS ACTIVITY OF ETHANOL EXTRACT OF GREEN ALGAE (*Spyrogyra* sp) *Purified* WITH *Chorio Allantoic Membrane* (CAM) METHOD

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Abstract

Background. *Angiogenesis plays an important role in tumor progression. Modern treatment of cancer currently has an alternative way 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.*

Objective. *This study aims to determine the antiangiogenesis activity of ethanol extract of green algae (Spyrogyra Sp) Purified by using the method of chorio allantoic membrane (CAM).*

Methods. *In this study used 8 groups: Group I was a control that content only paper discs, paper discs contain a group II + PBS, group III contain a paper disc + PBS + bFGF, group IV, V, VI, VII, VIII as a treating were given 10, 20, 40, 160, and 320 ug / ml of ethanol extract of green algae respectively. CAM is Obtained from embryonated chicken eggs aged 8 days. Incubated for 3 days afterward. The macroscopic observation is used to see the inhibitory activity of blood vessels. Statictical analysis used Kruskal-Wallis test to compare among each other Significantly, continuated by Mann Whitney test (P <0.05).*

Outcome measured. *Inhibitory activity (IC_{50})*

Results. *The results showed that the ethanol extract of green algae purified with concentration 10 ug / ml (p = 0:03) can inhibit angiogenesis of embryonated chicken eggs Compared to bFGF control.*

Conclusion. *In conclusion, the ethanol extract of green algae has antiangiogenesis activity in chorio allantoic membrane.*

Keywords : *antiangiogenesis, CAM, green algae, Spyrogyra Sp*

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INTRODUCTION

Cancer is a disease characterized by the uncontrolled proliferation of cells and the cells can invade and damage other part of bodies. The disease is still one of the leading causes of death in developed countries and the second leading cause of death in developing countries (Ahmedin, *et al*., 2011).

Melatonin is an alkaloid compounds and potent as inhibitors of cancer activity (Veronique *et al*., 2004). Melatonin works with three action in inhibiting cancer activities, which working with specific cytotoxic, slow cleavage (*proliferation*) of Cancer cells, and negate the toxic effects of heavy metals (epigenetic carcinogens). Mechanism of the latter action is because melatonin can stimulate cancer cells to synthesize *metallothioneins* (MTs) (Imbesi *et al*., 2008). These compounds are intracellular proteins that can protect cells by removing toxic effects of metals (*metal*) And other epigenetic agents (Korkmaz *et al*., 2008). Melatonin produced by the pineal gland in the human body and vertebrate animals. However, melatonin production in the body is very low (less than 100 mg) (Lerner *et al*., 1960). Based on this, it is necessary to melatonin intake from outside the body to inhibit the activity of the cancer and to eliminate the effects of toxic substances epigenetic.

Green algae (*Spirogyra sp*.) is a plant with a *phytomelatonin* content (Kolar and Machackova, 2001). *Phytomelatonin* is compound *melatonin* contained in the plant (*phyto*). Levels of compounds *phytomelatonin* in green algae at 240 ug / kg wet weight (Kolar and Machackova, 2001). *Phytomelatonin* in green algae can be isolated by using thin layer chromatography (TLC) (Lerner *et al*., 1960). Usefulness of these compounds can be seen as a barrier to cancer activity (*cancer inhibitor activity*) To test the cytotoxicity and antiproliferative test. Cytotoxicity test using the IC_{50} parameters or the concentration of the active substance that kills cells by 50% from the baseline, whereas the antiproliferative test using the parameter *doubling time* or length of time required by the cell in order to splitting into two times of the original amount (Katzung, 1995).

This study aims to investigate anti-angiogenesis effect of ethanol extract of purified green algae. Purification is done to obtain the fractions that containing of more melatonin and separated from the other components. By knowing how to purify green algae and determine the activity of anti angiogenesis then be developed the ethanol extract of green algae with melatonin standardized. This standardized extract may be developed as an anti angiogenesis in cancer therapy.

METHODS

Tool :The tools used in this study include the chamber chromatography, silica gel GF 254, the Soxhlet set, watch glass, rotary evaporator, glass objects, incubator, mini drill, LAF, camera, injection syringes, Eppendorf, micropipette, scissors and tools glassware.

Ingredients: Materials used in this study is the ethanol extract of green algae (*Spirogyra sp*.) Were extracted by the sohxlet method in Chemistry Laboratory of Pharmacy Faculty, Ahmad Dahlan University, bFGF, chorio allantoic membrane (CAM) of chicken eggs with 8 days ages, *paper disc*. Other chemicals were 96% ethanol, antiseptic solution, PBS, ethyl acetate, petroleum ether, and sterile aqua .

Green Algae Extract preparation: Green algae plants cleaned and put in a container filled with water, allowed to stand for 24 hours: 12 hours of irradiation phase and 12 hours of phase embezzlement. Algae taken from the container after 5-6 hours of embezzlement phase and then dried under the sun with a black cloth covered. Algae pollinated, pollen extracted by Soxhlet device using 96% ethanol. Pollen was evaporated to obtain thick extract.

Ethanol extract purified preparation: A total of 1000 mg of ethanol extracts obtained added with 15%acetic acid as much as 15 ml.

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The solution then filtered by using filter paper and a Buchner funnel. The filtrate was extracted with 15 ml of petroleum ether 3 times. Acidic water fraction added NH4OH until become pH 10, then extracted again with ether. Sari ether evaporated until obtained the thick extract. Test solutions were made in PBS solution.

Identification of Melatonin on green algae: Fraction of the ether which is obtained from thick extract spotted on silica gel KLT plate using capillary tube. The first spotted is the standard phytomelatonin, second spotted is purified of ethanol extract and third spotted is ethanol extract. Further silica gel GF 254 plates petted with a mobile phase *n*-Butanol-acetic acid-water (12:3:5). The results identified under UV light (254 nm).

Preparation of bFGF as an inductor of angiogenesis: Created stock levels of 50 ng /mL using a solution of 10 mM Tris-HCl pH 7.5 and then diluted to obtain a level of 0.5 ng / mL. BFGF levels are given for each treatment induced egg is 10 ng (Sun *et al*., 2004).

The inhibition of angiogenesis test: SPF chicken eggs that have been purchased, age 0 day immediately incubated in a laboratory incubator at a temperature of 390c. After eggs is 8-9 days old, the embryos location known through *candling* the egg. Egg shell at the pole containing the air space and egg shell above the embryo disinfected with iodine solution. Later in the two areas was made drill a small hole using a mini. The air from air chamber Aspirated with a rubber ball to move from the shell poles to the top of the egg. This treatment is done with egg horizontal position, in the dark, and through *candling*, and then the artificial air space formed in the embryo can be seen. Egg shell above the embryo was cut with a saw (mini drill) to make a rectangular window with an area of ??1x1 cm. Through this window, bFGF and isolates test was implanted into the *chorio allantoic membrane* (CAM). Eggs subjects test were divided into 8 groups (each treatment consisted of 4 eggs), as follows:

1) Group 1 is the egg with the implantation of paper disc.
2) Group II group with implantation of paper disc + Solvent (PBS) 10 mL.
3) Group III bFGF control group + solvent is the group of eggs with the implantation of the *paper disc* which is to content 10 ng bFGF + solvent (PBS) 10 mL.
4) Group IV, V, VI, VII and VIII are eggs used to see the inhibitory effect of the test solution with levels of variation is equal to 10 ug / ml, 20 ug / ml, and 40 ug / ml, 160 ug / ml and 320 ug / ml extracts.

Eggs in this treatment group were given implantation *paper disc* and added 10 ng bFGF and test solutions of polar fractions of green algae with concentration various by 10 mL.

Once treated, the eggs were incubated at a temperature of 37-39 ° C with a relative humidity of 60% for 3 days or 72 hours (Ribbati *et al*., 1997), then the egg is opened (age 12 days) and the contents of eggs laid. After that *chorio allantoic membrane* (CAM) which is attached to the shells was observed macroscopically and counted the number of new blood vessels grow on the surface and the edge of the paper disc. Macroscopic observation can be done with the aid of a magnifying glass (Jennie *et al*., 2006).

Data Analysis: Parameters observed in the study is the number of new blood vessels at the edges and surfaces of *paper disc*. To reduce the subjectivity of observations, then the observations made by three people, so the result is an average of the three observations. Observation of new blood vessels that form on the edges and surfaces of *paper disc*, must be distinguished from the main vein / origin of CAM. Where the main vein in CAM has a larger size, while the new blood vessels are arteries finer / smaller (Ribatti et al., 1999). After that the data obtained were analyzed statistically. Results of quantify the amount of new blood vessels can be calculated % inhibition by using the formula:

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$$\% \text{ Inhibition} = \frac{\sum \text{control blood vessel of bFGF} - \sum \text{treatment blood vessel}}{\sum \text{New blood control vessel}} \times 100\%$$

Results quantify the amount of new blood vessels then statistically analyzed using the Mann Whitney and Kruskal Wallis method (p = 0,05).

RESULTS AND DISCUSSION

Identification of melatonin Compound on ethanol extract purified

Test compound is a ethanol extract compound purified because it was partitioned by using several solvents in order to obtain extracts whose composition is almost pure. The stage of purification performed with ethanol extract and added 15% acetic acid to its alkaloid content tends to acidic. Subsequently partitioned with Petroleum Ether. Acetic acid extract was taken and added NH4OH to pH 10. Then the solution was extracted with ether and the solvent was evaporated to obtain a purified extract.

Identification of purified ethanol extract was carried out by thin layer chromatography method. On the identification of stationary phase used was silica gel GF254 while mobile phase used was a mixture of n-butanol ratio: 15% acetic acid: water (12:3:5). Identification results can be seen in Figure 1. Based on the identification can be seen that the ethanol extract of purified yield results spotted amount less than the ethanol extracts alone and active content of compounds melatonin in the ethanol extract is purified still look dominant.

Parameters on thin-layer chromatography is *retardation factor* (Rf), a comparison of the distance of analyte from the origin to the distance from the point of origin of the mobile phase. In the result it shows one spot on the standard and some spots on the samples, but one sample patches have the same Rf price with the Rf price of standard patches 0.68. This suggests that in the test solutions there are melatonin compounds.

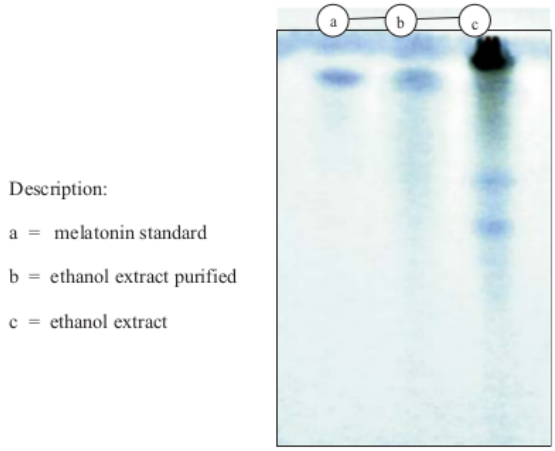

Figure 1. Qualitative identification of the ethanol extract, ethanol extract purified by comparison melatonin. = Silica gel stationary phase and mobile phase = 254 n-butanol: 15% acetic acid: water (12:3:5)

Rf values 0,68 on samples compounds are different due to different and vary levels of polar compounds. Polar compounds with polar stationary phase will be retained on the stationary phase. It can also be caused due to the non-polar compounds with non-polar mobile phase will elute higher than compounds that are more polar. In the picture looks not just one spot melatonin had with the test compounds but also there are some spots that appear on the elution results, it can be caused due to a lack of purification so that not only melatonin fraksinated. Such compounds may be able to support or reduce the activity of melatonin as an anticancer so we need more research to determine the contained of compounds in the purified ethanol extract.

Antiangiogenesis test

Angiogenesis is the formation of new blood vessels from existing blood vessels. The formation of blood vessels occurs either due to the effects of physiological and pathological

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effects, one of which occurs in cancer cells. Cancer cells secrete bFGF to form new blood vessels, so the research is conditioned as in cancer cells. bFGF is one of the pro-angiogenic factor that plays a role in the process of new blood vessel formation. bFGF is used as an inductor of angiogenesis in the test object. BFGF induction for each egg is equal to 10 ng. This dose has given the differences significant growth of blood vessels when compared with controls (Sun *et al*., 2004). The results of the calculation the average number of blood vessels in each control group and the group fed ethanol extract of green algae purified can be seen in Table I.

Table I. The results of the calculation of average number of blood vessels in each treatment group.

Treatment group	The average number of blood vessels (X ± SD)
Paper disc	0
PBS	0
bFGF	20:00 ± 0
10 ug / ml	4:33 ± 0:57
20 ug / ml	3.67 ± 12:57
40 ug / ml	2.67 ± 12:57
160 ug / ml	1:33 ± 0:57
320 ug / ml	1:00 ± 0:57

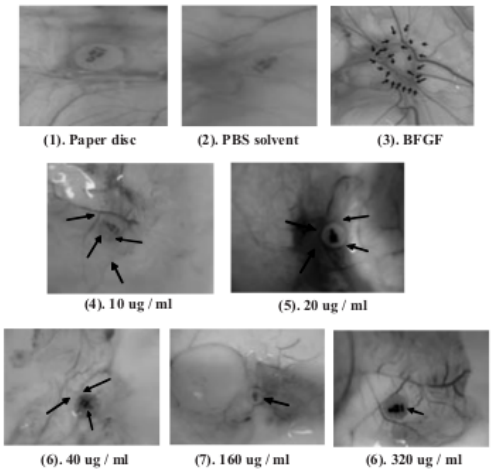

Figure 2. Observations macroscopically CAM

Macroscopically observations indicate that the test compound is purified ethanol extract could inhibit the growth of new blood vessels or angiogenesis in the CAM induced by bFGF. Test results angiogenesis purified ethanol extract showed the higher levels, the higher the inhibition of angiogenesis that occurs in bFGF-induced CAM is characterized by a decrease in the density of new blood vessels score. On eggs with test compound concentrations of 10 ug / ml seen the growth of new blood vessels in the area around the paper discs were decreased when compared with controls bFGF. The growth of new blood vessels is experiencing a reduction in the concentration of 20 ug / ml, 40 ug / ml, 160 ug / ml and 320 mg / ml.

Price% inhibition can also be obtained by finding the difference between the number of blood vessels in the bFGF and control the amount of blood vessels in each test solution concentration, divided by the number of blood vessels in the control group bFGF and multiplied by 100%. Price% inhibition of purified ethanol extract is shown in Table II.

Table II. % (percent) inhibition of purified ethanol extract

Concentration of Test Solution	% Inhibition of Blood Vessels (X ± SD)
10 ug / ml	78.33 ± 2.89
20 ug / ml	81.67 ± 2.89
40 ug / ml	86.67 ± 2.89
160 ug / ml	93.33 ± 2:22
320 ug / ml	95.00 ± 00:00

Purified ethanol extract have antiangiogenesis activity with the highest percent inhibition at a concentration of 320 ug / ml. Percent inhibition proportional to the concentration of the test solution, the higher the concentration of the test solution % inhibition of the higher percent.

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Test Result Statistics

In this study, the data obtained were normally distributed, but the data are not homogeneous, so that the analysis followed by Kruskal Wallis and Mann Whitney. This analysis was conducted to determine differences in treatment between groups. Mann Whitney test results showed a significant difference between groups. Here is a summary of the results of the Mann Whitney test for each treatment group.

Comparisons between the control group *paper disc* with bFGF control group showed significantly different results. This suggests that bFGF used in the study actually influence the process of angiogenesis. In contrast to the results of the analysis between the control group *paper disc* with PBS control. The analysis showed that there was no significant difference between the two treatment groups, so it can be concluded that the PBS solvent used in the study does not give effect to the formation of new blood vessels.

In the ethanol extract purified with various concentration 10 ug / ml, 20 ug / ml, 40 ug / ml, 160 ug / ml and 320 ug / ml showed significantly different results with the significance of bFGF control which means it can provide as an antiangiogenesis effect, this directly proportional to the inhibition of the growth of new blood vessels at every level variations in the concentration of ethanol extract purified.

In this study, purified ethanol extract shown to have activity as an inhibitor of angiogenesis inhibition mechanism itself, although it is not certain. Mechanism of inhibition of new blood vessel formation by purified ethanol extract test solution can not be determined related to the method used, the limitations of the method *chorio allantoic membrane* (CAM). This method is only aware of the antiangiogenesis effects based on the number of existing blood vessels after treatment, while the other responses that influence the angiogenesis inhibitor compounds can not be known. However, the mechanism of inhibition of a compound that may occur to the formation of new blood vessels associated with angiogenesis mechanism itself, among other mechanisms of inhibition of angiogenesis is the inhibition of invasion, motility, and cell adhesion, inhibition of endothelial cell activation, interfere with angiogenic growth factors or their receptors,

Table III. Summary of statistical analysis of antiangiogenesis test group and the control group treated with a test compound using *Mann-Whitney* test.

Variation	Control *paper disc*	BFGF control	PBS control	10 ug / ml	20 ug / ml	40 ug / ml	160 ug / ml	320 ug / ml
BFGF control	S							
PBS control	TS	S						
10 ug / ml	S	S	S					
20 ug / ml	S	S	S	TS				
40 ug / ml	S	S	S	S	S			
80 ug / ml	S	S	S	S	S	S		
160 ug / ml	S	S	S	S	S	TS		
320 ug / ml	S	S	S	S	S	S	TS	

Description: S = Significant contrast / contrast Meaningful (p = 0.05),
Unlike TS = Not Significant / Contrary Not Meaningful (P> 0.05)

inhibition the enzymes involved in the process of angiogenesis, and *vascular targeting* (Liekens *et al*., 2001).

In this study, which gives the effect of antiangiogenesis compounds in purified ethanol extract allegedly not only indicated by the presence of melatonin in the compound tested solutions but can also be supported by the presence of other compounds contained in the test solution. This can be seen from the results shown on the identification of melatonin has been done but more research needs to be done to determine the types of compounds contained in the test solution.

CONCLUSION

Ethanol extract of purified compounds containing melatonin have antiangiogenesis effect on *chorio allantoic membrane* (CAM) of chicken egg-induced *basic fibroblast growth factor* (BFGF) levels begin 10 ug / ml. The higher concentration of ethanol extract of purified, the higher the inhibition angiogenesis.

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