ETHANOLIC EXTRACT OF PASAK BUMI LEAVES ANTIANGIOGENIC ACTIVITY ON CHORIOALLANTOIC MEMBRANE OF CHICKEN EMBRYO INDUCED BY bFGF

Nina Salamah
Pharmacy Faculty, Ahmad Dahlan University
syifaniputri@yahoo.com
081804487736

Abstract

Background. *Angiogenesis, the growth of the blood vessel, allows cells to get enough supply oxygen and nutrient. Some study on natural substances have been conducted including pasak bumi leaves (Eurycoma longifolia, Jack)as anticancer. The aim of this study was to know the antiangiogenic effect of pasak bumi leaves using CAM method.*

Method*. The ethanolic extract of the pasak bumi leaves was prepared using maceration methode. The 9 day incubated chicken embryo were divided into seven groups. The group I was the control group received paper disc, group II was the bFGF controller, group III was the bFGF controller and DMSO 0,8% as the solvent. The test groupreceived different concentration (65 ug/ml, 130 ug/ml, 260 ug/ml, and 520 ug/ml) of extract ethanolof pasak bumi leaf in combination with 10 ug bFGF . After having been incubated for three days, the content in the egg was taken out. Then, the corio allantoic membrane attaching the eggshell was carefully observed macroscopically and microscopically.The result of macroscopically observation was quantified and analyzed.*

Result. *Based on the result of the study, the ethanol extract of pasak bumi leaves can actually inhibited angiogenesis on CAM induced by bFGF.*

Conclusion. *Ethanol extract of pasak bumi leaves showed antiangiogenic activity began to level 130 mg/ml and increasing concentrations of ethanol extract of pasak bumi leaves enhance angiogenesis inhibitory activity.*

Keywords : *angiogenesis, Eurycoma longifolia, pasak bumi, CAM, bFGF*

ISBN : 978-979-18458-5-4

INTRODUCTION

Angiogenesis is the formation of new blood vessels from pre-existing ones, and it allows the cells to get nutrients and oxygen supply, therefore it can continues to survive (Hanahan & Weinberg, 2000; Folkman, 1996). There is presumption if there are chemical agents that have ability to impede neo vascularization, then the chemical agent have potential in therapeutic treatment of various diseases, including cancer (Ribbati et al., 2002). Therefore, the identification and characterization of the factors of angiogenesis proangiogenic and inhibitors is very important in cancer treatment.

Up to now, the utilization of natural medicine as an alternative cancer drug is being widely explored. Pasak bumi (Eurycoma longifolia Jack) is native plant from Indonesia which has the potential anti-cancer (Sengupta et al., 2004). The extracts of Methanol, butanol, chloroform, and water from the roots of pasak bumi shown to have cytotoxic effects on cells of KB, DU-145, RD, MCF-7, CaOV-3, and MDBK (Nurhanan et al., 2005). Other studies have shown that the methanol extract of the roots of pasak bumi was sitotosik on HeLa cell cultures with values IC50= 46,9 - 58,6 ?g/ml (Mustofa dan Qamariah, 2004). Several studies have been done with focus on the root of pasak bumi, therefore it also important to know the potential of the pasak bumi leaves. This study has aim to identify the antiangiogenic activity of ethanol extract of leaves of pasak bumi in the CAM of chiken embryo which induced by bFGF.

METHODS

Materials

Leaves of Pasak bumi (Eurycoma longifolia Jack) obtained from the Banjar Baru, South Kalimantan with 90% ethanol extraction solvent.

Angiogenesis inductor used in this study was the recombinant of human bFGF 1ng/?l which was purchased from Sigma with the production number F 0291. Chorio allantoic membrane (CAM) of chicken embryo derived from SPF chicken eggs were 8 days, purchased at the Veterinaria Farma Center Surabaya. Other chemicals are DMSO 0,8 %, buffer solution Tris-HCL 10mM pH 7,5, etanol 70 %, formalin 10%, NaCl 0,9%, aquadest steril and hemaktosilin-eosin (HE).

Research Procedures

The method of extraction is using maceration of powder leaves of pasak bumi 200 gram with 500 ml ethanol solvent 90 %. The ethanol solvent for 24 hours was filtered with a Buchner funnel. The maceration was repeated in 5 times and the ethanol extract is evaporated to obtain viscous extract

Anti-Angiogenesis Test

All equipments that were used to test anti-angiogenesis are sterilized by autoclav, temperature of 1210 C for 15-30 minutes. Preparation of basic fibroblasts growth factor (bFGF) are used as much as 25 ?g, and stocked on levels of 50 ng / ml Tris-HCL 10 mM 7,5 pH then diluted to obtained levels of 1 ng / ml. Preparation of bFGF was performed aseptically in a laminar air. BFGF doses given for each treatment-induced egg are 10 ng (Sun et al., 2004)

Ethanol extract of pasak bumi leaves (E. longifolia) was dissolved in DMSO- distilled water 0.8% sterile and then made into some level series. Subsequently is sterilized using micro filter. Preparation was carried out aseptically in a laminar air flow.

SPF chicken eggs aged 8 days (incubation) immediately incubated again in a laboratory incubator at a temperature of 39 ? C. Test subjects of eggs were divided into 7 groups (each treatment group consisted of 5 eggs) as follows: Group I is acting as the control group received paper disc, group II as the bFGF controller, group III as the bFGF controller and DMSO 0,8% as the solvent. group II with the egg induced is the egg by the implantation of paper disc that containing 10 ng bFGF. The test group

IV, V, VI, and VII received different concentration (65 ug/ml, 130 ug/ml, 260 ug/ml, and 520 ug/ml) of extract ethanol pasak bumi leaves in combination with bFGF 10 ug.

Once treated, the eggs were incubated at 39 ? C with relative humidity of 60% for 3 days or 72 hours (Ribbati et al., 1997), and then the egg is opened (age 12 days) and the content of the eggs ejected. Egg was opened by cutting the shell into 2 parts, starts of the shell that close to the air cavity, afterward the chorio allantoic membrane which is attached to the shells observed in macroscopic and microscopic.

Macroscopic observation was carried out by magnifying glass, while the microscopic observations carried out on histological preparations of the egg CAM (Jenie et al., 2006)

Parameters observed in this research is the number of new blood vessels or angiogenesis response in the CAM after treatment of ethanol extract of leaves of the pasak bumi (E. longifolia). The data obtained were statistically analyzed with the Kruskal Wallis and Mann Whitney test. Microscopic observation was made by observing the histological preparations of CAM

Anti-angiogenesis test was conducted using method who proposed by Ribatti et al. (1997) which has been modified by Jenie et al. (2006) which is not use gelatin sponges as a carrier test preparation but using sterile paper disc. Paper disc has its disadvantages, especially relating to the ability of absorption. Moreover the observation of new blood vessels disrupted by the presence of blood vessels from the paper disc implanted and the presence of non-specific inflammatory reaction, it causes difficulties when quantification of angiogenesis response. However, these modifications are then controlled by adding one variable that the control group of paper disc to minimize the weakness.

RESULT AND DISCUSSION

Based on the results of statistical analysis of control group showed that between the control paper discs and bFGF control there were significant differences, and between the control group bFGF and the solvent control group there were no significant differences. These data support the results of macroscopic observations above that show bFGF is an appropriate inductor angiogenesis in CAM method and the DMSO solvent was not affect and still can be used.

The next control group was the control group of paper disc and the inductor treatment of bFGF to determine the potential inductor to the emergence of new blood vessels in and around the paper disc. In the process of test compounds it used DMSO solvent 0.8% therefore it was need to know whether the solvent effected on the observations in CAM or not. Afterward is performed the control of the paper disc which is given inductor bFGF and added the solvent. Macroscopic observations of the three controls are presented in Table I and Figure 1.

Tabel I. Observation results of angiogenesis on control group

Control group	N sampel	Score
paper disc	4	+1
paper disc + bFGF	4	+3
paper disc + bFGF + DMSO 0,8 %	4	+3

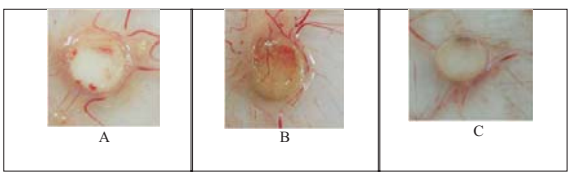

Figure 1. Angiogenesis Response of control group

A.*paper disc,* B. *paper disc*+ bFGF, C. *paper disc*+ bFGF + DMSO 0,8%.

Macroscopic observation of the CAM paper disc control group, showed that angiogenesis in the paper disc, and the area around the paper disc shows a similar picture

with the angiogenesis on overall CAM, it means that the paper disc can be used as a carrier in this method, because it did not affect to CAM angiogenesis. In the control group of bFGF it Based on these observations, it appears that the solvent used does not affect to the CAM, and then it can continue to use. The results above are supported by the data in Table I.

Tabel II. Angiogenesis Response on control bFGF + pelarut and Test Group etanol extract pasak bumi leaves

Test Group	N	Score
control bFGF + DMSO 0,8%	4	+3
bFGF + ethanol extract concentration 65 ?g/ ml	4	+3
bFGF + ethanol extract concentration 130 ?g/ ml	4	+2
bFGF + ethanol extract concentration 260 ?g/ ml	4	+2
bFGF + ethanol extract concentration 520 ?g/ ml	4	+1

seen a lot of new blood vessels branching from the CAM blood vessels were formed earlier and shown in the paper disc and the area around the paper disc. Base on the findings it means bFGF could induce angiogenesis. There were a lot of

The research provide five series levels of ethanol extract of leaves at 65, 130, 260, and 520 ug / ml. Anti-angiogenesis data of ethanol extract of pasak bumi leaves shown in Table II and Figure 2

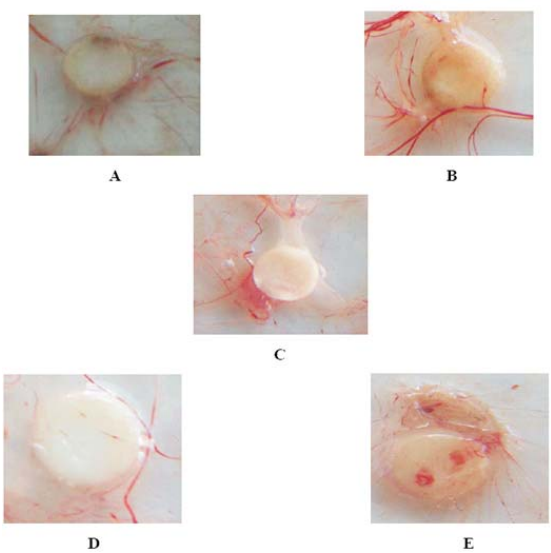

Figure 2. The Macroscopic observation of control group bFGF+DMSO 0,8 % (A) and Test group (B/65 ug/ml,C/130 ug/ml, D/260 ug/ml, E/520 ug/ml).

new blood vessels form in the paper disc and the area around the paper disc with a radial pattern.

The results of macroscopic observation shows that the higher levels of the extract, the greater inhibition of angiogenesis.This can be

ISBN : 978-979-18458-5-4

seen from the density of blood vessels which were decreased in the paper disc and around the paper disc. Treatment of ethanol extract of 65 mg / ml did not show inhibitory activity of angiogenesis. The effect become seen when the treatment used 130 ug / ml. The results of Kruskal Wallis test indicated that there were significant difference of angiogenesis inhibition between the control and ethanol extract test groups with 4 series. However, the Mann Whitney test showed that there was no significant difference of angiogenesis inhibition between the levels of 130 ug/ml and 260 ug/ml of extract with +2 score. Only at levels of 520 ug / ml of extract there was significant difference inhibition with a score of +1. These test results indicated that the ethanol extract has potential as an anti-angiogenesis.

Microscopic examination of angiogenesis response was conducted to determine more clearly the changes that occur in blood vessels of CAM due to extract treatment and compared with the microscopic picture of the control group who were given the cancer inductor and the DMSO solvent. The microscopic pictures were only to support the results of macroscopic picture.

The figure 3 shows the changes that occurd in blood vessels of CAM as a treatment group when compared to the control group. In the control group, it was seen that the numbers of new blood vessels are more frequent when compared to the treatment group. The picture illustrating that the extract of pasak bumi leaves has anti-angiogenesis activity in a certain degree.

Based on this research, it was known that ethanol extract of pasak bumi leaves could inhibit angiogenesis even though still has not yet certain about the mechanism of inhibitory action. The release mechanism of bFGF as a growth factor is the bFGF interacts with endothelial cells through a tyrosine kinase and heparan sulfate proteoglycan (HSPGs) receptor on the cell surface. The balance between storage and release of bFGF in the extra cellular matrix is an arrangement biological effect of these growth factors in the endothelium. Angiogenesis is a complex process, where there is a balance situation between pro-angiogenic and antiangiogenic factors. To inhibition of angiogenesis, the neutralization of one of the factors pro-angiogenic (bFGF) is sufficient to disrupt the balance of angiogenesis, and it will leading to the inhibition (Ribatti, 2002)

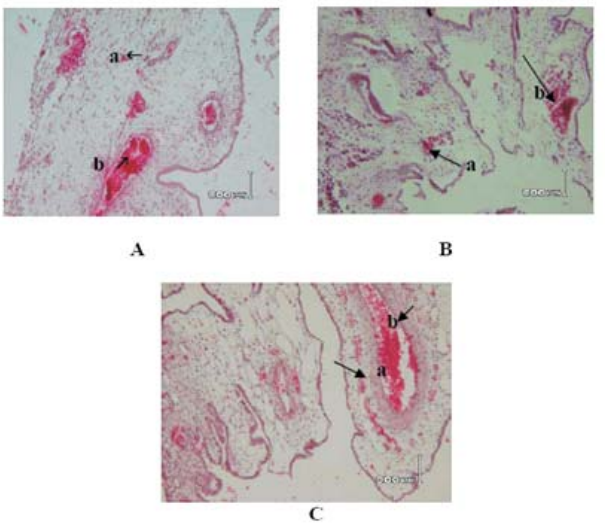

Figure 3 . The Microscopic observation of Angiogenesis Response CAM perbesaran 10 x 10
a. new blood vessel, b. first blood vessel
(A) Ethanol extract pasak bumi leaves consentration 65 ug/ml, (B) 260 ug/ml dan (C) control bFGF+pel

Research on anti-angiogenesis in the quasinoid compounds has not been done so the inhibition mechanisms still not yet clearly known. Inhibition of angiogenesis is an attractive offer for the target of therapy with low toxicity. Based on a low mutation rate in the genetic stability of endothelial cells, antiangiogenic therapy is the first step for specific tumor therapy (Kleinsmith et al., 1999). Targeted cancer therapy with the directly inhibition of angiogenesis in endothelial cells (the cells forming new blood vessels) is also likely related to the mechanism of targeted cancer therapies, such as inducing apoptosis of endothelial cells, inhibits endothelial cell proliferation and inhibit metastasis because of new blood vessels to be used for the metastasis of cancer cells are not formed.

Research on the compounds effects of the methanol extract of pasak bumi root isolation in inducing apoptosis of HeLa cell cultures was conducted by Nurkhasanah and Pihie (2004) which was likely related to the mechanism of inhibition of angiogenesis directly on endhotelial cells that inhibits migration and proliferation that caused the new blood vessels. In this process, if there is an endhotelial cells migrate off there is possibility to inhibit through of apoptosis mechanism so that endhotelial cells can not proliferate to form new blood vessels. Inhibition of blood vessel formation is likely also plays an important role in inhibiting the metastasis because of new blood vessels also become one way for the metastasis of cancer cells. There is the possibility of metastatic cancer cells in other ways, namely via the lymphatic system, but it still needs further study, whether the methanol extract in this study have the ability to inhibit metastasis via lymphatic vessels.

CONCLUSION

Ethanol extract of pasak bumi leaves showed antiangiogenic activity began to level 130 mg/ml and increasing concentrations of ethanol extract of pasak bumi leaves enhance angiogenesis inhibitory activity.

REFERENCES

Anonim, 1989, Materia Medika Indonesia, Jilid V, Departemen Kesehatan Republik Indonesia, hal 212-215.

Folkman, J,1996, Fighting Cancer by Attaching its Blood Supply, *Scientific American* 19: 116-119

Giavazzi, R., Albini, A., Bussolino, F., DeBraud, F., Presta, M., Ziche, M., Costa, A., 2000, The biological basis for antiangiogenic therapy (meeting report), *European Journal of Cancer.,* 36: 1913-1918.

Hanahan, D and Weinberg, R.A., 2000, The Hallmark of Cancer, *Cell.,*100:57-68.

Jenie R.I., Meiyanto, E., Murwanti, R., 2006, Efek antiangiogenik ekstrak etanolik daun sambung nyawa (*Gynura procumbens* (Lour.) Merr pada membran korio alantois (CAM) embrio ayam, *Majalah Farmasi Indonesia*, 17(1):50-55

Kleinsmith, L.J., Kerrigan, D, Kelly., 1999, Angiogenesis, accessed on http://press2.nci.nih.gov/sciencebehing/angiogenesis/angio00.html.

Knighton, D., D. Ausprunk, D.Tapper, and J. Folkman, 1977, Avascular and Vascular Phases of Tumor Grrowth in The Chick Embrio. Br.J. Cancer. Cek penulisannya 35 no. 347-356.

Mustofa dan Qomariah N., 2004, Aktivitas Antiplasmodial *in vitro* dan Sitotoksik Akar Pasak Bumi terhadap malaria di Kalimantan Selatan, Medika, 3, 147-152

Nurhanan M.Y., Hawariah L.P.A, Ilham A.M., Shukri, M.A.M, 2005, Cytotoxic Effects of the Root Extracts of *Eurycoma longifolia,* Jack,*Phytother. Res.* 19:994-996

Nurkhasanah and Pihie A.H.L., 2006, Apoptotic Cell Death Induced by Eurycomanone (Eurycoma longifolia, Jack) in Human Cervical Carcinoma Cells, Proceeding of

International Conference on Mathematics and Natural Sciences

Ribatti, D., Gulandaris, A.,Bastaki, M., Vacca,A., Lurlalo, M., Roncali, L., Presta, M.,1997, New Model for Study of angiogenesis and Antiangiogenesis in The Chick Embryo Chorioallantoic Membrane: The Gelatin Sponge/ Chorioallantoic Membrane Assay, *J Vasc Res*, 34: 455-463.

Ribbati, D., Vacca, A., Presta, M., 2002, The discovery of angiogenic factors: A historical review*, General Pharmacology.,* 35:227-231.

Sengupta, S., Toh S.A., Sellers L.A., Skepper, J.N., Koolwijk, P., Leung, H.W., Yeung H.W., Wong, R.N.S., Sasisekharan R., Fan, T.P.D, 2004, Modulating Angiogenesis : The Yin and the Yang in Ginseng, *Circulation.* 110:1219-1225

Sun, X., Ding, Y., Yan, X., Wu, L., Li, Q., Ceng, N., Qiu, Y., Zhang, M., 2004, Angiogenic synergistic effect of basic fibroblast growth factor and vascular endothelial growth factor in an *in vitro* quantitative microcarrier-based three-dimensional fibrin angiogenesis system, World J Gastroenterol 2004; 10(17): 2524-2528