Antiangiogenesis from Pericarp of Mangosteen on T47D Breast Cancer

Lusiana Arifianti *, Siti Rofida, Sukardiman, Noor Cholies Zaini
Departement of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Airlangga University
Corresponding author: +6281931016555, Surabaya, Indonesia, l.arifianti@gmail.com

Mangosteen (*Garcinia mangostana* Linn.) are generally cultivated in tropical rain forest area in Southeast Asia, especially in Indonesia. Mangosteen are well known to be rich in xanthone compounds and have anticancer activity. This study was aimed to know the mechanisms of action as an anticancer agent, such as an angiogenesis inhibitor on the T47D breast cancer cell line by suppressing angiogenic factor (VEGF) in vitro from the ethanol extract and actives fractions of mangosteen pericarp. The materials test of this study were the ethanol extract of mangosteen pericarp and fractions 2, 2.2 and 2.2.4 that generated from separations on previous study. In this study, we determine the antiangiogenic potency on T47D breast cancer through determination of expression percentage of VEGF as angiogenic factor by imunocytochemistry assay method. The result showed that the ethanol extract and the active fractions possesed antiangiogenic potency and the fraction of 2.2.4 was the most potent with the lowest percentage of VEGF expression (24,67% ± 4,51). It can be concluded that chemical components that contained in the extract and the fractions of mangosteen pericarp gived angiogenesis inhibition on T47D breast cancer cell. Based on this result, the ethanol extract and the active fractions of mangosteen pericarp were potential to be delevoped as antiangiogenic agent in breast cancer metastase.

Key words:, *Garcinia mangostana*, angiogenesis, VEGF, T47D, Breast Cancer

BACKGROUND

Breast cancer is the commonest cancer among women and the second highest cause of cancer death. Most cases occur during age 45–55. It also occurs in men but is more than 100-fold less frequent than in women. The lethality of breast cancer is largely due to metastasis, preferentially to the lymph nodes, lungs and bones. In order to delay the progression of breast cancer and prolong patient life, more effective chemopreventive and antimetastatic treatmentts and less toxic chemotherapeutic agents are desperately required (Hondermarck, 2003).

Garcinia mangostana Linn or mangosteen is known as "the queen of fruits", belongs to Guttiferrae (Clusiaceae) family. These plants are generally cultivated in tropical rain forest area in Southeast Asia , such as Indonesia, Malaysia, Thailand and Sri Lanka and commonly harvested in the dry season (Hung *et al*., 2009). Mangosteen fruit has a sweet taste and a little sour, while the pericarp is dark red, and a 2/3 parts of the fruit are generally discarded as trash. The pericarp rich in xanthon and anthocyanin contents, and Southeast Asian peoples traditionally used it to treat diarrhea, trauma, and skin infections (Lim *et al*., 2012).

The chemical constituents of mangosteen are xanthon, flavonoids, triterpenoids, benzophenone, biphenyl compounds, pirrole, benzofuran, tannins and saponins. While xanthon is the major secondary metabolites in mangosteen (Chin and Kinghorn, 2008).

Previous studies have shown that the compounds of mangosteen pericarp as cytotoxic agent on SKBR3 and MCF-7 breast cancer cells, and Hep2 laryngeal cancer (Moongkarndi *et al*., 2004; Chitra *et al*., 2010 and Ahmat *et al* 2010). It also indicated a potent anti-proliferative xanthons compounds from the pericarps of mangosteen against human leukemia HL60 cells which mitochondria as the preferential target (Matsumoto et al., 2004). Moreover, it induced cell-cycle arrest and apoptosis in human colon cancer DLD-1 cells (Matsumoto *et al*., 2005). However, it has not been known the ability of angiogenesis inhibition on T47D breast cancer cells by the extract and mangosteen fraction researches before.

Therefore, in this study we investigate the antimetastatic ability by suppressing VEGF as an angiogenic factor of crude ethanol extract and the active fractions from pericarps of mangosteen based on MTT cytotoxic assay (in previous study) from *Garcinia mangostana* by using T47D breast cancer cell line as a model to complite the data information about the potency of *Garcinia mangostana's* pericarp as an anticancer sources.

MATERIALS AND METHODS

Materials

Plant material. Mangosteen (*Garcinia mangostana*) were obtained from Blitar, in East Java, Indonesia. The material tests were ethanol extract of mangosteen pericarp and ethyl acetate fraction(F2), and its sub fractions (F 2.2 and F 2.2.4) which the IC_{50} of ethanol extract, F2, F 2.2 and F 2.2.4 were 8.96 ppm; 1.79 ppm; 1.75 ppm and 1.12 ppm respectively).

Cell line. Human breast cancer cell lines, T47D derived from the CCRC (Cancer Chemoprevention Research Center), Gajah Mada University, Yogyakarta. It was maintained in RPMI 1640 (Gibco, Invitrogen Company) containing 10% Fetal Bovine Serum (FBS) (Sigma), 2% Penicillin-Streptomycin (Sigma) and 1% Amphotericine B (Sigma) 37°C in a humidified atmosphere of 5% CO2 and 95% air (Eppendorf). Cells were checked routinely and found to be free of contamination.

Chemical materials. Dimethyl sulfoxide (DMSO) (Sigma), PBS (Phosphate Buffer Saline) (Sigma), ethanol 70% (-20°), Novostatin Universal Detection Kit, VEGF Antibody monoclonal primer (Invitrogen).

Methods

Imunocytochemistry assay. Cells ($5x10^4$ cells/well) were transfered to coverslips (Nunc) in 24-well plate (Iwaki) and incubated for 24 hours (70-80% confluent). Cells were treated by ethanol extract, fraction 2 (F 2), fraction 2.2 (F 2.2) and fraction 2.2.4 (F 2.2.4) of mangosteen pericarp, and then incubated for 15 hours. At the end of the incubation, coverslips containing cells were moved to object glass. Acethon (E.Merck) was added to fixated the cell. Then, the normal mouse serum (1:50) was added and wait for 15 minutes. Dropped it with the VEGF polyclonal antibody (Bioss) (1:50), and wait for 60 minutes. Washed it by PBS three times. And then incubated in biotin for 10 minutes, and washed by PBS two times. After that, incubated the preparate in *streptavidin-peroksidase* (Lab Vision) for 10 minutes, and washed by PBS two times. Then, incubated in chromogen-3,3-diaminobenzidin/ DAB (Lab Vision) for 3-8 minutes, and washed by aquadest. Soaked into haematoxylin (Dako) for 3-4 minutes (counterstain purpose) and washed by aquadest. The protein expression can be observated by light microscope (Olympus CKX21). The VEGF expression showed by brown or dark stain on the cell, while blue/purple showed the normal cell (300 population of the cells) (Master and John, 2000; Fresney, 2005).

RESULTS AND DISCUSSION

First we investigate the identification of chemical compounds of ethanol extract and fractions from mangosteen pericarp by using thin-layer chromatography method (Densitometer Camag Scanner 3) with silica gel F254nm as stationary phase and n-hexane: chloroform: methanol = 5:5:1 as a mobile phase. The results showed that all of them containing α-mangostin as compared to the standard α-mangostin. Thus it can be expected that α-mangostin compound contain in the extract and fractions of mangosteen pericarp that gived dominantly contribution to the anticancer activity.

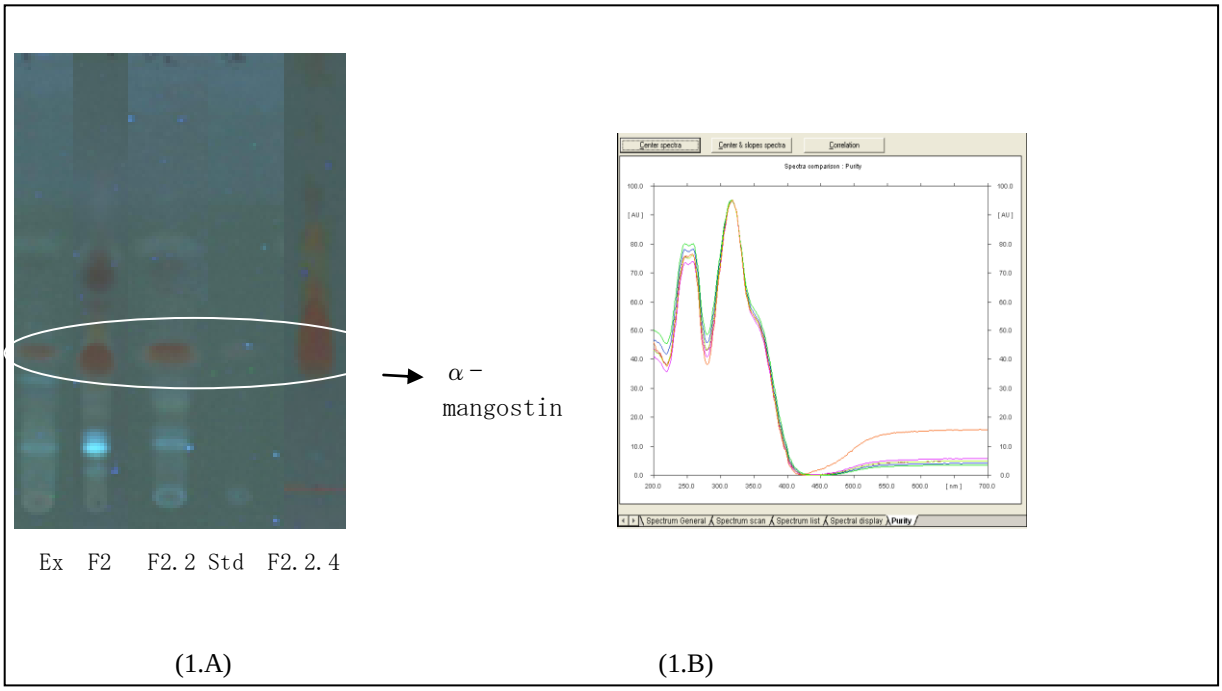
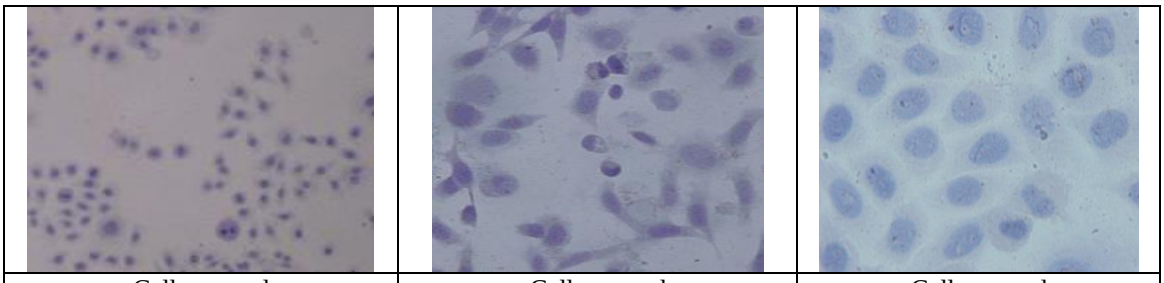


Figure 1.A Thin layer chromatography plate imaged by TLC Camag visualizer on 365nm wave length all of the samples (ethanol extract, F2, F2.2 and F2.2.4 while compared with α-mangosten standard. **1.B** Overlay of the α-mangostin spectra of ethanol extract, F 2, F 2.2 and F 2.2.4 while compared with α-mangosten standard.



Cells control (negative VEGF)(100x)	Cells control (negative VEGF)(400x)	Cells control (negative VEGF)(1000x)

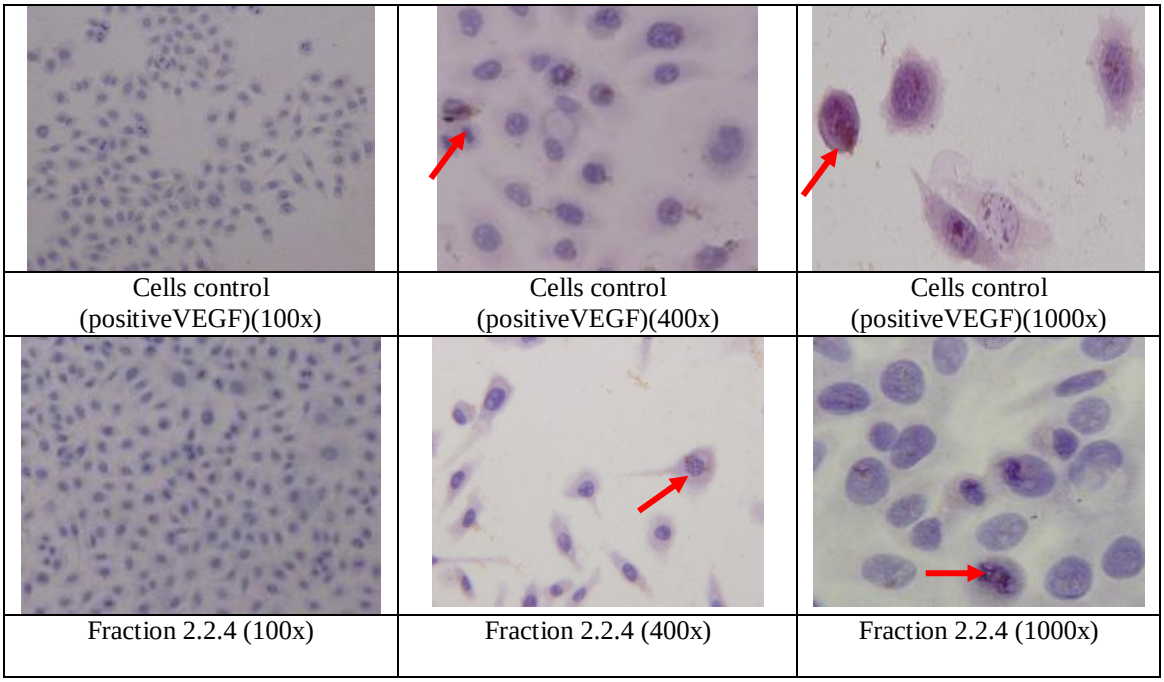


Figure 2. Determinate of VEGF expressions on T47D breast cancer cells. Cells gived brown spot on the cells when expressing VEGF protein. The examinations were observed by light microscope Olympus CX on varies magnified (100x; 400x and 1000x).

The main causes of death in patients with breast cancer was due to the presence of cancer metastases. To grow and metastasize, the cancer cells need blood vessel formation through angiogenesis. Angiogenesis is the physiological process by which new blood vessel capillaries are formed. It proceeds rapidly during normal tissue development but occurs in adulthood only during very specific periods, such as wound healing and formation of the placenta during pregnancy. Angiogenesis hapened by stimulation of angiogenic factors such as VEGF (Vascular Endhotelium Growth Factor) and bFGF (Basic Fibroblast Growth Factor) on endothelial cells to secrete some enzymes protease and plasminogen activator (PA) enters the cell and invade the surrounding matrix, resulting in degradation of the vessel basal membrane. Furthermore, endothelial cells migrate, proliferate and differentiate into new lumen containing blood vessels (Hung *et al*., 2009; Tiwari, 2012).

One way to detect the process of angiogenesis by using immunocytochemistry method. Immunocytochemistry is a method used to detect the expression of a specific protein or antigen in cells by using specific antibodies that will bind to the protein or antigen. Its involves incubation of cells with antibodies. Antibodies will bind to specific antigens or proteins in the cell. Free antibodies separated by washing, while the binding antibodies directly detected with labeled primary antibody, or indirectly by enzyme-labeled secondary antibody (Meiyanto *et al*., 2006).

Therefore, in this study we determined the expression of VEGF protein as a marker of angiogenesis support, and the ability of suppressing VEGF expression can indicates that the test substances were antiangiogenic agent.

Table 1. Percentage of VEGF expression of each samples treatments on T47D breast cancer

	Percentage of VEGF expression (%±SD)
Ethanol Extract	39.67 ± 1.53
Fraction 2	39.00 ± 5.66
Fraction 2.2	35.67 ± 0.58
Fraction 2.2.4	24.67 ± 4.51

* ($p \geq 0.05$)

The determination of the VEGF expression percentage in each treatment showed that the extract and active fractions mangosteen pericarp provide lower VEGF expression compared with control cells. This means that the chemical components in pericarp success to suppress the expression of VEGF, as an angiogenic factor that can cause and increase in cancer metastasis. One-way ANOVA statistical analysis (SPSS 17.0) gived different percentage of VEGF expression ($p \geq 0.05$), unfortunately the post hoc test (statistical analysis (SPSS 17.0) indicated that the extract group, fraction 2 and fraction 2.2 were not significantly different except fraction 2.2.4 ($p \geq 0.05$), this suggests that the ability to suppress the expression of VEGF in the extract, fractions 2 and fractions of 2.2 are considered equal while Fraction 2.2.4 is the most potent fractions in suppressing VEGF with the percentage of VEGF expression only 24.76 % ± 4.51.

Thus, the ethanol extract and active fractions of mangosteen pericarp can inhibit angiogenesis through suppression of the VEGF expression as angiogenic factors. It consistent with the previous study, reported that α-mangostin has inhibiting effect on the antimetastase with road adhesion, migration and

invasion of cells by cell-matrix adhesion assay method, wound healing and boyden chamber assay against prostate cancer cells PC-3 expression via suppression MMP-2 and MMP-9 (Hung *et al*., 2009). MMP-2 and -9 are proteinase enzyme that regulate invasion and metastasis, through fibrin molecules degradation formed by endothelial cells and basal membrane. While VEGF is a supporting factor for the formation of new blood vessels. So, it can be said that VEGF and MMP synergy in the development of angiogenesis (Yang *et al*., 2011).

CONCLUSION

This study concluded that chemical components contained in the extract and the fractions gived angiogenesis inhibition on T47D breast cancer cell. Based on this result, the ethanol extract and the actives fractions of mangosteen pericarp were potential to be delevoped as antiangiogenic agent in breast cancer metastase.

REFERENCES

Ahmat, N., Azmin, N.F.N., Ghani, N.A., Aris, S.R.S., Sidek, N.J., Abdullah, S., Jasmani, H., 2010. Bioactive Xanthones from The Pericarp of *Garcinia mangostanan*. ***Middle-East Journal of Scientific Research***, Vol. 6(2), p. 123-127

Chin, Y.W., and Kinghorn, A.D., 2008. Structural Characterization, Biological Effect, and Synthetic Studies on Xanthones from Mangosteen (*Garcinia mangostana*), a Popular Botanical Dietary Supplement. ***Organic Chemistry,*** Vol. 4(4), p. 355-364.

Chitra, S., Krithika M.V., Pavithra, S., 2010. Induction of Apoptosis by Xanthones from *Garcinia mangostana* in Human Breast Cancer and Laryngeal Carcinoma Cell Lines. ***Int J of Pharma and Bio Sci,*** Vol. 1(3), p. 1-8

Fresney, R.I., 2005. ***Culture Of Animal Cell*** : A Manual of Basic Technique. 5th Ed. John Wiley and Sons

Hondermarck, H., 2003. Breast Cancer. ***Molecular & Cellular Proteomics 2.5.*** The American Society for Biochemistry and Molecular Biology, Inc. pp. 281-291. http://www.mcponline.org accessed on 8th May 2012

Hung Shun-Hsing, Shen Kung-Hung, Wu Cheng-Hsun, Liu Chang-Liang, Shih Yuan-Wei, 2009. A Mangostin Suppresses PC-3 Human Prostate Carcinoma Cell Metastasis by Inhibiting Matrix Metalloproteinase-2/9 and Urokinase-Plasminogen Expression trough the JNK Signaling Pathway. ***Jour of Agr and Food Chem***, Vol. 57 (4) , p. 1291-1298

Lim, T.K., 2012. Garcinia mangostana. In:***Edible Medicine and Non Medicine***, Vol. 2 Fruts, Lim, T.K. (Edt). Dondhrect Holdberg London New York: Springer Science and Business Media BV, p. 83-108

Masters and John R.W., 2000. ***Animal Cell Culture***: A Practical Approach. 3rd Ed. 3rd Floo Research Laboratories, University College London: Oxford University Press

Matsumoto K, Akao Y, Yi H, Ohguchi K, Ito T, Tanaka T, Kobayashi E, Iinuma M, Nozawa Y., 2004, Preferential target is mitochondria in alpha-mangostin-induced apoptosis in human leukemia HL60 cells***, Bioorg Med Chem***., 12(22):5799-5806

Matsumoto, K., Akao, Y., Ohguchi, K., Ito, T., Tanaka, T., Linuma, M., Nozawa, Y., 2005. Xanthones induce cell-cycle arrest and apoptosis in human colon cancer DLD-1 cells. Bioorganic & Medicinal Chemistry,Vol. 13, p. 6064-6069

Meiyanto, E., Melannisa, R., Da'I, M., 2006. PGV-1 decreases angiogenic factor (VEGF and COX-2) expression on T47D cell induced by estrogen. ***Majalah Farmasi Indonesia***, 17 (1), pp. 1– 6

Moongkarndi P, Kosem N, Kaslungka S, Luanratana O, Pongpan N, Neungton N., 2004, Antiproliferation, antioxidation andinduction of apoptosis by Garciniamangostana (mangosteen) on SKBR3 human breast cancer cell line, ***J. Ethnopharmacol***., 90(1):161-166

Tiwari, M., 2012. Apoptosis, Angiogenesis and Cancer Therapies. ***Jour. of canc ther and res***, pp. 19

Yang, S., Zhao, Z., Wu, R., Lu, H., Zhang, X., Wu., X, and Guan., G., 2011. Expression and biological relationship VEGF-A and MMP in gastric carcinoma. ***The Jour. of Int. Med. Res,***Vol.39, pp. 2076-2085