

Antiangiogenic Effect of Ethanol Extract of *Citrus Reticulata* Peel In The Chorio Allantoic Membrane (CAM) Induced by bFGF

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Abstract

One of the characteristic of cancer is new blood vessels formation or angiogenesis. Angiogenesis supplies oxygen and nutrition for cancer cells in order to fulfill their needs to keep growing. So, blockade of angiogenesis is a promising strategy to suppress tumor growth, invasion, and metastasis. Tangeretin and nobiletin which concentrated in the peel of citrus fruit are widely known has anticancer effect. In this study, the antiangiogenic effect of ethanol extract from the *Citrus reticulata* peel was determined. The CAM (Chorio Allantoic Membrane) methods was used for this aim. Eggs at the age of nine days were divided into 7 groups. Two groups are control: paperdisc and bFGF+vehicle. The next five groups are ethanol extract of citrus fruit peel that variate in 5 dossage: 75, 150, 300, 450, dan 600 µg which applicated into paper disc. At the age of twelve, macroscopic and microscopic analysis was done. Macroscopicly, the extract group could inhibit the new blood vessels formation. This fact was supported by microscopic analysis. Based on haematoxylin-eosin staining, macrophage cells in the extract group was less than the bFGF+vehicle group. These two analysis suggest that the ethanol extract of *Citrus reticulata* peel can perform as antiangiogenic agent.

Key words : Angiogenesis, *Citrus reticulata* peel, tangeretin, nobiletin, macrophage

Introduction

Angiogenesis is an important step in cancer growth. Angiogenesis which appeared in cancer cell is a neovascularization that happen in abnormal condition. New blood vessels is needed to supply oxygen and nutrition into cancer cells, so that cancer cells can keep growing, developing or even metastating. Based on this evidence, angiogenesis can be said act as main concept of cancer growth (King, 2000). Therefore, vascularization can be potential target in cancer therapy.

To date, antiangiogenesis therapy is considered, worldwide, as a promising approach, supposedly leading to the desperately needed

breakthrough in cancer therapy and other proangiogenic diseases. Even though many antiangiogenic therapies for treating cancer were only highly active in animal models, but at clinical results so far are disappointing (Griffioen and Molema, 2000). Hopefully using natural product can resolve these problem (Cassady *et al.*,1990).

The peel of citrus fruits is a rich source of flavanones, as well as many polymethoxylated flavones, which are very rare in other plants (Choi, 2007). Two of many compounds that contained in *Citrus reticulata* peel are tangeretin and nobiletin. Tangeretin and nobiletin can up-regulate p53 expression. Previous research found that tangeretin could inhibit HL-60 leukimic cell line growth (Hirano *et al.*, 1995). Bracke *et al.*, in 1999 reported that tangeretin inhibited the invasion of MO4 cells into embryonic chick heart fragments in vitro. But, research about antiangiogenesis activity of Citrus peel extract has not been done yet.

In this study, the antiangiogenic effect of ethanolic extract of *Citrus reticulata* peel was examined. This research is important to develop *Citrus reticulata* as anticancer agent. This research is also useful for enrich information about ethanolic extract of *Citrus reticulata* peel that suggested has anticancer activity by angiogenesis inhibition.

Materials And Methods

Citrus reticulata was collected from Tawangmangu-Indonesia. The peels were collected, dry and extracted using ethanol 96%.

Antiangiogenesis observation

Incubated embrio chicken Chorio Allantoic Membrane (CAM) Spesific Pathogen Free (SPF) at the age of nine, Recombinant human bFGF (Invitrogen), ethanol 70%, sterile aquadest.

Methods

Observation of chemical constituent of Ethanolic extract of Citrus reticulata peel

This observation was done to find out that there is a group of compound which has anticancer activity: flavonoid (Ren *et al.*,2003). Normal phase chromatography was used with silica gel GF254 as solid phase and ethyl acetate-methanol-water (100:13,5:10) as mobile phase (Wagner and Bladt, 1996).

Antiangiogenesis observation

Modified Corio Allantoic Membrane (CAM) method was used in this study (Ribatti, 1997). Fertilized white leghorn chicken eggs at the age of seven were incubated at 37⁰ C within 2 days. Then, at the age of nine, a square of window was made in the shell (1x1 cm) by using

a wheel bit on a dentist drill. Through this window, a paper disc which contained bFGF and extract was implanted to the embryo. Eggs were divided into several groups: paper disc, bFGF+vehicle (30 ng bFGF+PBS+co-solvent DMSO) and bFGF+vehicle+extract group in several doses: 75, 150, 300, 450, 600 μg (Bracke *et al.*, 1999), which were applied on the 19,64 mm² square paper disc. Extract was prepared by extracting plant material obtained from Balai Penelitian Tanaman Obat (Jl. Raya Lawu Tawamangu, Center Java).

Following paper disc implantation, eggs were incubated at 37^o C at 60% humidity within 72 hours (Ribatti *et al.*, 1997). At the age of twelve, eggs were opened and removed its component. Then, the chorionic membrane that patched on the egg's surface was observed macroscopically and microscopically. This observation was documented by camera.

Result

Observation of chemical constituent of ethanolic extract of *Citrus reticulata* peel

Flavonoid is a group of compounds which contained in *Citrus reticulata* peel (Nickavar, 2006). Normal phase chromatography was used with silica gel GF254 as stationary phase and ethyl acetate-methanol-water (100:13,5:10) as mobile phase (Wagner and Blatt, 1996). From figure 1, blue colour under the UV365 light could be visualized. This indicated that ethanolic extract of *C. reticulata* contains flavonoid.

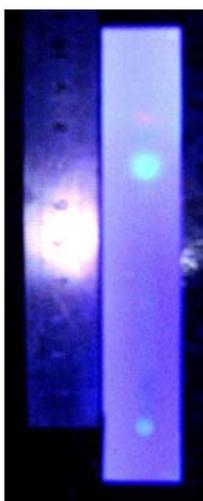


Figure 1. TLC profile of ethanolic extract of *Citrus reticulata* peel

Antiangiogenesis observation

Macroscopic and microscopic observation were performed to determine angiogenic response. Corio allantoic membrane macroscopic observation showed that the appearance of blood vessels on the paperdisc and surround the paper disc were not the same as angiogenesis formed on CAM surface (Fig. 2). The blood vessels on the paper disc were thinner than surround the paper disc. This indicated that bFGF could stimulate new blood vessels formation on CAM.

Microscopic observation was shown in figure 3. Using haematoksilin-Eocin staining, epithel cells which in granular shape was appear in pink colour. Macrophages were also shown in this staining (Fig. 3)

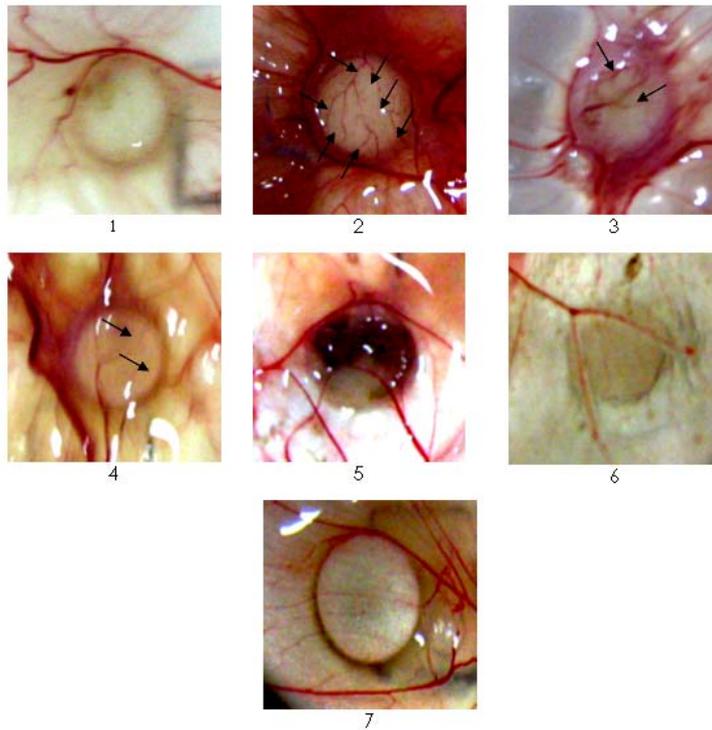


Figure 2. Angiogenesis response of control and extract group on embrio egg CAM at the age of 12 after 3 days incubation. Number 1 and 2 are paper disc and bFGF+vehicle group. Number 3-7 are bFGF+vehicle+extract group which divide into 5 dose: 75, 150, 300, 450 and 600 µg that applicated on the paper disc. Black arrows show new blood vessels growth

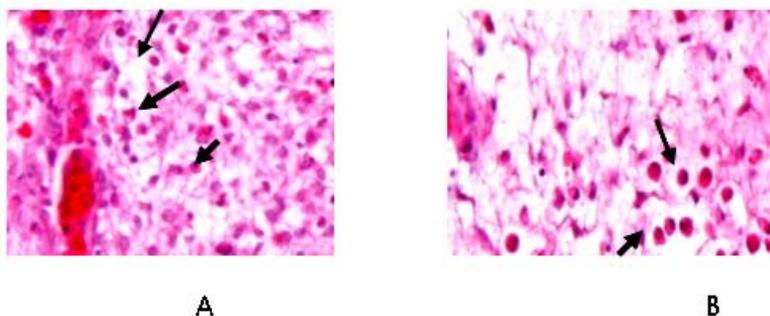


Figure 3. Microscopic observation by Haematoxylin-Eosin staining (A) bFGF+vehicle dan (B) bFGF+vehicle with ethanolic extract of *Citrus reticulata* peel in 75 µg dose. Black arrows show macrophages

Discussion

Observation of chemical constituent of ethanolic extract of *Citrus reticulata* peel

Flavonoid is a group of compound which contained in *Citrus reticulata* peel (Nickavar, 2006). Under the UV365 light, flavonoid will appear yellow, blue or green fluorescence depends on the structure (Wagner and Bladt, 1996). Based on the chromatographic analysis, it can be concluded that flavonoid compound is found in ethanolic extract of *Citrus reticulata* peel.

Antiangiogenesis observation

Flavonoid is widely known has anticancer activity. One of the mechanism how flavonoid inhibits cancer cells growth is through angiogenesis inhibition. In 1997 and 1993, Fotsis *et al* found that hydroxylated flavonoid can inhibit angiogenesis *in vitro*. The other class of flavonoid, polymethoxyflavone (PMF) which highly found in citrus peel, was reported to give higher anticancer activity (Choi *et al.*,2007; Attaway, 1994). Many research has been done to find out anticancer activity of PMF (Walle, 2007). But, the antiangiogenesis activity of Citrus peel extract has not been done yet.

On the untreated paper disc, angiogenesis formation can not be seen (Fig.2). Meanwhile, on bFGF+vehicle gives angiogenic effect significantly. This shows that 30 ng bFGF is effective on the angiogenesis induction. Extract in 75 µg still shows angiogenic effect. But, the amount of new blood vessel formation is less than bFGF+vehicle. This antiangiogenic effect is proportional with the amount of the extract that applicated in paperdisc. In 150 µg extract also shows that the extract can inhibit old blood vessel growth. This effect is also given by 300 µg extract. In 450 and 600 µg extract, angiogenesis can not be observed at all. Even, in 600 µg extract also

can inhibit old blood vessels that pass on the paper disc. This was showed by the thickness of the old blood vessels that pass on the paper disc is less than ones pass beside the paper disc. This suggest that ethanolic extract of *Citrus reticulata* peel can inhibit angiogenesis formation in dose dependent manner. Even though, quantitative analysis is needed to measure the antiangiogenic effect by ethanolic extract of *Citrus reticulata* peel.

The antiangiogenic effect showed maybe caused by tangeretin and nobiletin which are contained in the extract. Tangeretin and nobiletin are PMF compound that have more amount than the other PMF in the *Citrus reticulata* peel. The amount of tangeretin and nobiletin in *Citrus reticulata* peel are 124 and 110 mg per 100 g wet weight (Nogata *et al.*,2006). Tangeretin was known can up-regulate p53 expression and CDK inhibitor: p21 and p27 (Pan *et al.*,2002). p53 plays an important role in angiogenesis. Overexpression of p53 can up-regulate thrombospondin-1 (TSP-1) which act as angiogenesis inhibitor (Holmgren *et al.*,1998; Yuan *et al.*,2002). p53 also inhibit VEGF expression by suppressing the VEGF mRNA production that mediated by TATA BOX binding on VEGF gene (Yuan *et al.*,2002; Tai *et al.*,2002). Because of that, more research needed to see whether ethanolic extract of *Citrus reticulata* peel can up regulate p53 expression on CAM.

Angiogenesis inhibition by extract also can be caused by suppressing Matrix Metalloproteinases-1 and -9 (MMP-1,-9) production and up-regulating Tissue Inhibitor of Metalloproteinases (TIMP) which performed by nobiletin. On the previous research, nobiletin can suppress MMP-1,-9 production and up-regulate TIMP on Human Fibrosarcoma HT-1080 cancer cell line (Sato *et al.*,2002). Nobiletin also reported can suppress prostaglandin E2 (PGE2) production and cyclooxygenase-2 (COX-2) expression *in vitro* (Murakami *et al.*,2000). COX-2 was observed has a role in angiogenesis (Bakhle, 2001). Beside that, COX-2 can be inhibited by tangeretin-p53 mediated (Subbaramaiah *et al.*,1999). Further research required to see the down-regulating effect of MMP-1,-9 and COX-2 production and also to see the up-regulating of TIMP production which performed by ethanolic extract of *Citrus reticulata* peel.

On the microscopic observation, can be seen that the number of macrophage given by the 75 µg extract is less than bFGF+vehicle control. Macrophages was known can secrete a number of angiogenic factor like VEGF, bFGF and Interleukin 8 (IL-8) which can induce angiogenesis and TNF-α that increase the number of VEGF, bFGF and IL-8 receptor (Lee *et al.*,2006; Koch *et al.*,1992; Yoshida *et al.*,1997) (Fig. 4). IL-8 action in angiogenesis is induced by its ability in stimulating of MMP-2 production (Luca *et al.*,1997). Therefore, the less of macrophage means the less angiogenic factor and its receptor

that produced. The less of these macrophages may be caused by cell cycle arrest that induced by tangeretin-p53 mediated.

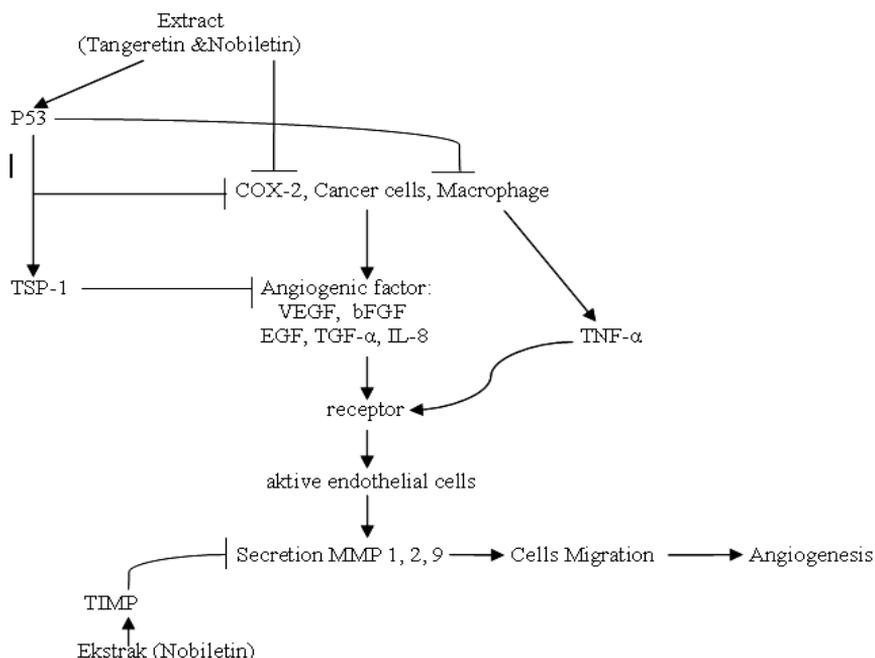


Figure 4. The suggested mechanism of antiangiogenic effect of ethanolic extract of *Citrus reticulata* peel

Summary

Ethanolic extract of *Citrus reticulata* peel can perform as antiangiogenic agent. Even though, further research to see p53 control in angiogenic factor like MMP-1, -2,- 9; IL-8; TIMP dan VEGF need to be done.

Conclusion

Ethanolic extract of *Citrus reticulata* peel has antiangiogenic effect on Corio Alantoic Membrane induced by bFGF.

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