Ferulic Acid Prevents Angiogenesis Through Cyclooxygenase-2 and Vascular Endothelial Growth Factor on The Chick Embryo Chorioallantoic Membrane Model

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INTRODUCTION: This study designed to verify the antiangiogenic activity of ferulic acid (FA) and its potency to inhibit COX-2 and VEGF expression on CAM model. Moreover besides, we verified its mechanism of action by docking the molecule on COX-2, tyrosine kinase and VEGF-2 proteins in silico
METHODS: Anti-angiogenesis assay of FA at the doses of 30, 60 and 90 µg were performed using CAM of chicken eggs with nine-day old which were stimulated by 60 ng basic fibroblast growth factor (b-FGF). Celecoxib 60 µg was used as reference drug. The inhibitory activity on VEGF and COX-2 expressions were conducted by immunohistochemistry assay (IHC). Molecular docking of FA were accomplished by Molegro Virtual Docker program ver. 5.5. on COX-2 enzyme (PDB ID 1CX2), tyrosine kinase receptor (PDB ID 1XKK) and VEGF-2 receptor (PDB ID 4ASD).
RESULTS: FA at doses 30, 60, 90 µg significantly prevented angiogenesis on CAM model ($p<0.05$), which were represented as inhibitory activities against endothelial cell of blood vessels (42.6-70.7%) and neovascularization (43.0-86.6%). Inhibitory activities of FA against VEGF expression were stronger than its action on COX-2 expression. Molecular docking on VEGF-2 receptor result in RS value of FA was -73,844 kcal/mol, and celecoxib was -94.557 kcal/mol. RS value on tyrosine kinase of FA was -84.954 kcal/mol, while celecoxib was -93.163 kcal/mol. Docking on COX-2 receptor denoted RS value of FA was -73,416 kcal/mol, while celecoxib was -118,107 kcal/mol.
DISCUSSION AND CONCLUSION: Reduction of VEGF-2 & COX-2 expression due to treatment with FA at dose range 30–90 µg seem to be related to angiogenesis inhibition, which is presented by two parameters, namely inhibition of neovascularization and endothelial cell growth in blood vessels It was concluded that FA is promising anti-angiogenic therapeutic agent especially at early stage, and this activity can be resulted from inhibitory action on COX-2 and VEGF-2 proteins.

Keywords: ferulic acid, COX-2, VEGF, tyrosine kinase, angiogenesis, chorio allantoic membrane

Introduction

The progression of cancer cells depends heavily on angiogenesis, the formation of new blood vessels which supply oxygen and food required for tumor's cell proliferation and metastases progression[1,2]. The development of cancer cells is induced by angiogenesis which is activated by some growth factors such as vascular endothelial growth factor receptor (VEGFR). Two important growth factor receptors which influence angiogenesis are tyrosine kinase receptor (TKR) and VEGFR-2[1,3].

The vascular endothelial growth factor (VEGF) signaling pathway is a strategic target for the angiogenesis inhibitors[4,5]. But, there are some clinical problems of

angiogenesis inhibitors in patients (i.e. thromboembolic disorders, intracranial hemorrhagia, and bowel perforation)[6]. Other problems are the occurrence of toxicities such as bleeding, fatigue, hypertension, perforation in gastrointestinal tract[7,8]. Therefore, the study of new antiangiogenic substance which is relatively safe in their pharmaceutical dosage form is one of some important steps in cancer treatment.

Ferulic acid (FA), [3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid] (Figure 1) is a phenolic acid compound, which has antioxidant[9,10], and cytotoxic activities in many cancer cell lines[11,12]. Wang et al. (2016) reported that FA could inhibit proliferation and induced apoptosis of osteosarcoma cell via PI3K/Akt pathway[13]. FA also had anti-inflammatory activity and inhibited cyclooxygenase-2(COX-2) enzyme[14] which were related to angiogenesis[2,15]. Koki et al. (2003) reported that COX-2 expression increased during the tumorigenesis process and the intensity of its expression was in line with the degree of carcinogenesis[16]. Wu et al. (2006) reported that biosynthesis result of prostaglandin which was catalyzed by COX-2, could stimulate the vascular endothelial growth factor (VEGF) expression in tumor cells and acted as a positivefeedback loop between tumor cells and endothelial cells[17].

In an effort to improve FA as a drug candidate for cancer chemoprevention, we study its antiangiogenic activity and inhibitory activity against COX-2 and VEGF expression on the chicken egg chorioallantoic membranes (CAM) model. The CAMs assay is a robust and applicable technique to assess a potential drug candidate. The assay has been commonly used to study angiogenesis and tumor invasion in various type of cancer[18,19]. To verify FA's mechanism in inhibition of angiogenesis, we conducted molecular docking on COX-2 enzyme, tyrosine kinase,and VEGF-2 receptors.

Material and Methods

Materials

Commercially available materials were used as obtained. Ferulic acid, Tris-HCl and basic-Fibroblast Growth Factor (bFGF) were purchased from Sigma-Aldrich (St Louis, MO, USA). The chicken eggs were collected from PUSVETMA Surabaya, Indonesia.

CAM assay

Twenty-fiveof nine-day-old chicken eggs were incubated at 37ºC (60-70% humidity) for one day. All of the treatment groups of CAMs were induced by bFGF at a concentration of 1 ng/µl, in Tris-HCl solution of pH 7.5. The eggs were spread into five

groups; each group consists of six eggs. Three groups were treated with FA at dosages of 30, 60, 90μg; the positive control group was treated with celecoxib 60μgand one group without treatment which was set as the negative control. The egg was perforated to make a hole of 1 cm^2 in diameter to remove the air. The sterile Whatman filter-paper disks of 5-mm in diameter with tested compound and bFGF or bFGF alonewere dropped into the hole and then were impregnated onto CAM of each embryo. The hole was covered and restored to incubation at 37ºC (60% humidity) for 72 hours. After incubation, the upper eggshell was opened and the presence of neovascularization from the main blood vessels onto the paper disks were counted. In addition, we also collected the blood vessels of CAM in the formalin buffer and carried out microscopic observations of the histopathologic slide of the CAMusing Hematoxylin and Eosin (HE) staining. To confirm the antiangiogenic activity, this CAM assay was achieved with certain alteration as explained before[18,20]. The growth of the endothelial cell in the neovascular capillaries wasexposed in CAM cross-sections by an inverted phase contrast microscope, Nikon H600L. The number of endothelial cells at five graphical fields were calculated, whereas each slidewas observed at 400x amplification and compared with positive or negative control groups for the following analysis.

Antibody:

Rabbit polyclonal anti-humanCOX-2 and VEGF antibody were obtained from Thermo Scientific™ Lab Vision™ (St Louis, MO, USA).

Immunohistochemical assay

The slides for immunohistochemical assay were prepared at Laboratory of Anatomical Pathology, Dr. Sardjito Hospital, Yogyakarta, Indonesia. Paraffin blocks of the CAM containing blood vessels were cut in 3-4 μm thick and placed on the poly-lysine slide, then they were incubated overnight at 45ºC. The next step of VEGF or COX-2 staining for the immunohistochemical assay was carried out as reported in previous research[18,19]. In this study, we used Hematoxylin Mayer as counterstaining. The Tris-EDTA solution at pH 9 was used in the retrieval step of VEGF staining for 15-20 min, whereas pH 6 citrate buffer was used for retrieval COX-2 staining for 15-20 min. The slides divided into six groups. One group consist of slides of CAM expressing COX-2 protein which was used as positive control, three groups contain slides of FA at doses of 30, 60 and 90 μg, one group were slides of celecoxib at a dose of 60 μg which was

used as the reference drug, and one group was used as a negative control group. The microscopic observation of VEGF expression and COX-2 expression were continued by counting the visible brown cells at five fields for a minimum of 100 cells.

Statistical Analysis

The obtained data were determined to an analysis by ANOVA, one-way Fisher test (F) at 95% level of confidence. If there was a significant variance among each group, the analysis was continued by Duncan multiple range test. Statistical significant was stated if $P<0.05$. All statistical analyses were presented using IBM SPSS Statistic 21 program.

Molecular Docking Equipment

Hardware: Laptop ASUS AMD A8 Vision with CPU @1.9 GHz, 4GB of RAM

Software: CS ChemBio Draw Ultra version 12.00 (Cambridge Soft) was utilized to create the three-dimensional structure (3D) of FA which was then subjected to energy minimization by MMFF94 method and the 3D structure was saved as SYBYLMol2 format (*.Mol2). Program Molegro Virtual Docker (MVD) version 5.5 (CLC Bio) was employed as a docking program in this research. The 3D structure of COX-2, TKR, and VEGF-2 proteins were downloaded from website (http://www.rcsb.org/pdb/home /home.do).

In silico assay

The 3D structure of three proteins which were obtained from a protein data bank (PDB) were: 1) 1XKK, that was TKR which contains *N*-(3-chloro-4-[(3-fluorobenzyl)oxy]phenyl}-6-[4-quinazo-linamine) (code FMM_901) as reference ligand, 2) 4ASD, that was VEGFR-2 which contains sorafenib (BAX 43-9006) as reference ligand, and (3) 1CX2, that was COX-2 which contains 4-(5-(4-bromophenyl)-3-(trifluoro-methyl)-1H-pyrazole-1yl)benzenesulfonamide) (SC58) as reference ligand. Ligand-protein interactions were prepared by FA molecule MVD version software 5.5. Docking of FA molecule into each of the 3D structure of proteins was performed as described in our previous study[20]. The best docking results can be noticed by relating the structure of the docked FA molecule with the crystal structure of reference ligand in the binding site. The docking results were presented as a rerank score (RS), which was the total free energy of ligand-protein interaction. The lowest energy indicated the best pose of binding between the functional group of ligand and amino acid residues

of the proteins (Table 3-5). The ligand-protein complexes of the lowest score (RS) were used for further visual inspection (Figure 5-7). The validation of the docking result was performed by redocking the reference ligand into the same cavity in each protein. Redocking result was accepted if root means square value (RMSD) < 2.0 Aº.

Results and Discussion

Antiangiogenic Activity of FA

The anti-angiogenesis assay is performed on the CAM of the embryonated chicken egg because the membrane is the site of very high vascularization and easily observable in line with the process of chicken embryo growth[18,21]. The CAM is a fusion of chorion and allantois of chicken egg membrane which is formed after 4 days incubation[18]. The formation of new blood vessels is derived from the capillaries that arise from the blood vessels.

Angiogenesis on CAM model through macroscopic observations of new blood vessels induced by b-FGF is significantly inhibited ($P<0.05$) by FA at dosage 30, 60, 90 μg. The results are represented by inhibition of neovascularization (Figure 2).

Neovascularization is inseparable from the preceding mechanism of growth and the formation of new endothelial cells, whereas one of the angiogenesis stages is endothelial cell migration[22]. At this stage, the capillary walls of the blood vessels continue their growth into the lumen of other blood vessels. There are four phases of migration: the growth of two opposite capillary walls, the joining of regulated endothelial cells and bending of the bilayer facilitate growth factors so the cells enter the lumen, the center of angiogenesis is formed between the new blood vessels filled by pericytes and myofibroblasts. And the center of angiogenesis is the appearance of smooth muscle fibers that complement the formation of new blood vessels with the development of hematopoiesis as early vascularization.

Figure 2 showed that several capillary blood vessels progressing into the impregnated disk of the group without treatment. After treatment with FA and celecoxib, there are a decrease in the number of new blood vessels around the disk on CAM and endothelial cell of blood vessels. Percentage of neovascularization and endothelial cells growth of blood vessels inhibitions were performed in Table 1.

Based on statistical analysis by one way ANOVA program, followed by Duncan multiple range test,it is known that there is no significant differences potency inhibition of neovascularization between celecoxib at dose 60 μg and FA at dosage 60-90 μg ($P<0.05$). But, potency FA at 30 μg is smaller than FA at 60-90μg. Whereas potency

FA at dose 90 μg inhibits endothelial cell of blood vessels is stronger than FA at dosage 30-60μg. No significant differences in the potency inhibition endothelial cell of blood vessels between FA at dose 90μg and celecoxib at dose 60μg (P<0.05).As a reference drug, celecoxib 60μg providing 83% of new blood vessel growth inhibition on the CAM model.

FA inhibited VEGF and COX-2 expression

The microscopic image of VEGF expression in the endothelial cell of the new blood vessels belongs to the positive control group is dominated by the presence of brown color. It is very contrast when compared with an image from the negative control group, it displays the dominant blue color in the endothelial cell cytoplasm. The brown color on the cytoplasm which indicates VEGF expression of the endothelial cell treated by FA decreases with increasing dosage of the substance from 30μg to 90μg. The microscopic observation results of VEGF expression in each treatment group presented in Figure 3.

The microscopic observation results of COX-2 expression in each treatment group presented in Figure 4. All data of the treatment group were presented in Table 2. and analyzed statistically by SPSS 21.0 version for Windows software.

Analysis of percentage inhibition of VEGF expression by FA, using the Normality One-sample Kolmogorov-Smirnov test showed probability or p = 0.398, whereas percentage inhibition of COX-2 expression with the Normality One-sample Kolmogorov-Smirnov test showed probability or p=0.458. This means the data is normally distributed because of greater than 0.05 (p>0.05). Based on statistical analysis by one way ANOVA program, continued with LSD and Duncan Multiple Range tests, it is known that there is no significant differences potency inhibition of VEGF expression between celecoxib at dose 60 μg and FA at dosage 60-90 μg (p<0.05). Potency FA inhibits VEGF expression at 30 μg to 90 μg was dose-dependent. Increasing dose of FA resulted in decreasing VEGF expression of CAM significantly.

Based on the data in Table 2, it is known that giving 30-90 μg FA decreases VEGF expression in CAM significantly. Reduced of VEGF expression due to treatment with FA at doses 30-90μg is in line with angiogenesis inhibition, which appears in two parameters, namely neovascularization or resistance to endothelial cell growth of blood vessels on the chick embryo chorioallantoic membrane.

The main VEGF receptor on endothelial cells is VEGFR2, which influenced during development and adult cells. The VEGFR2 signaling is better recognized than the other VEGF receptors. The vascular permeability is essential*in vivo* consequence of VEGFR2 activation. Several small-molecular-weight inhibitors of VEGFR2 kinase activity are employed clinically to block pathological angiogenesis in cancer[13].

In vitro experiments of capillary endothelial cells showed that VEGF is a powerful stimulator against the occurrence of angiogenesis. This is due to its presence as a growth factor triggering the proliferation and migration of endothelial cells, even the formation of tube formation in a series of capillary vessels[28]. Inhibition of VEGF can occur in several options, the first is inhibition at the VEGF receptor through kinase activity that contributes to the delivery of growth signals. The second possibility is inhibition through endothelial cell apoptosis induction, whereas the third may occur to the incorporation of hematopoiesis and endothelial progenitor cells, so VEGF is not capable of triggering vasculogenesis[30].

According to Giovana *et al*. (2009), binding of VEGF receptor (VEGFR) and VEGF-specific ligand occurs in the transmembrane and cytoplasmic domains. VEGF is known as a promoter of angiogenesis and an endogenous regulator of endothelial integrity. Some anti-VEGF compounds can cause endothelial dysfunction and decreased angiogenesis[29].

The COX-2 expression on CAM also presented the brown color in the cell cytoplasm which is a ligand-receptor complex of COX-2 detected by anti-COX-2 antibodies that are specifically bound to COX-2 ligand.Based on data in Table 2, it is known that treatment with 30–90µg FA can significantly decrease COX-2 expression in CAM. The inhibitory activity of FA against COX-2 expression is dose-dependent. The inhibitory potency of FA at dose range 30–90µg are significantly different from celecoxib at the dose 60µg. In our study, the administration of FA appears to play an important role in angiogenesis inhibition through decreasing in COX-2 expression.

Reduction of COX-2 expression due to treatment with FA at dose range 30–90 µg seem to be related to angiogenesis inhibition, which is presented by two parameters, namely inhibition of neovascularization and inhibition of endothelial cell growth in blood vessels of chick embryo chorioallantoic membrane. Hsu et al. (2017) described that prostaglandin-E2 (PGE2) stimulate COX-2 expression in LoVo colon cancer cells. The COX2 expression interrelated with the elevation in the migration ability of that cancer cells. The experimental data suggested that PGE2 can be a potential therapeutic target in colon cancer metastasis[29]. Yao et al (2011) informed

that down-regulation of COX-2 could significantly diminish the progression of gastric cancer cells, and blockade the replacement and tube formation of human umbilical vein endothelial cells. The results also presented that down-regulation of COX-2 might inhibit VEGF[25].

COX-2 inhibitor, Celecoxib[23], has been reported to inhibit the growth of prostate, gastric, lung, and breast cancer[24,25]. The induction of b-FGF as pro-angiogenic compounds will lead to an inflammation associated with the COX-2 activity[26,27]. As well as celecoxib, FA also inhibited COX-2 enzyme[14,28], so it could obstruct the b-FGF activity as a pro-angiogenic compound.

Based on the inhibitory mechanism of angiogenesis through the inhibition of VEGF expression conducted by other researchers in several studies, it is possible that FA inhibits VEGF receptor (VEGFR-2) through its inhibitory activity on tyrosine kinase and COX-2. This assumption is studied and proven through *in silico* docking on COX-2, TKR, and VEGFR-2.

Docking Study

The best-docked pose of FA, celecoxib, BAX ligands in the binding site of VEGFR-2 and the interactions between functional groups of FA and Celecoxib with amino acid residues in two dimensional (2D) structure are shown in Figure 5. The docking scores (RS value), the type of bonding interactions with VEGFR-2 and amino acids involved in their interactions were listed in Table 3.

The best-docked pose of FA, celecoxib, native ligand in the binding site of Tyrosine kinase and their interactions with amino acid residues in two dimensional (2D) structure are shown in Figure 6. The RS value, the type of interactions and amino acids involved in the interaction between ligand and tyrosine kinase receptor (TKR) were listed in Table4.

The ligand of PDB ID 1XKK contains a benzylic group (Ar-C-) which acts as a pharmacophore in the ligand-receptor interaction complex. The FA compound contains a similar group which is also expected as a pharmacophore. Celecoxib that produces lower RS value than FA (Table 3) display more H-bonds and steric interactions with TKR than FA.

The best-docked pose of FA, celecoxib, native ligand in the binding site of Cox-2 and their interactions with amino acid residues in two dimensional (2D) structure are shown in Figure 7. The RS value, the type of bonding interactions with COX-2 and amino acids involved in their interactions were listed in Table 5.

Based on molecular docking study, it can be explained the possible interactions between each compound, i.e. FA and celecoxib, with amino acid residues in each protein enzyme/receptors. The interaction FA-TKR is stronger than FA-VEGFR2, indicating possibility FA worked on growth factor receptors[1,31],and it is apparent that FA inhibited VEGF expression of CAM model.

The ligand binding of VEGFR persuades strong tyrosine phosphorylation and consequences in an intense angiogenesis response[31]. Replacement native ligand of VEGFR-2 with FA through the interactions functional groups of FA with the amino acid residue of VEGFR-2 prevented tyrosine phosphorylation, so decrease angiogenesis proses[32].

There are several phases of angiogenesis inhibition, i.e. prevention of endogenous angiogenic factors such as b-FGF and VEGF; inhibition the degradation matrix metalloproteinase (MMP), reducing endothelial cell proliferation; prevention endothelial cell movement and inhibits endothelial cell activation and differentiation[33].

Based on the data and description above, the mechanisms FA prevent angiogenesis are by blocking COX-2 activity so that MMP activity which plays a role in the early formation of branching of blood vessels will stop and by obstructing an endogenous angiogenic factors, i.e. VEGF and b-FGF in initially stage of angiogenesis.

Study Limitations on our study, FA inhibits angiogenesis on CAM model in the earliest process, i.e. endogenous angiogenic factors and kinase activity that promotes to release of growth signals. Thus, potency FA inhibits angiogenesis at other stages need further research.

Conclusions

Ferulic acid (at dosage of 30-90μg) is a promising anti-angiogenic therapeutic agent, especially in an early stage of angiogenesis process. It prevents pro-angiogenic growth factor which leads to inflammation (i.e. b-FGF), the activity of MMP and development of branching of blood vessels. This activity result from inhibitory action on COX-2 and VEGF-2 proteins.

Ethics approval and consent to participate

This assay was approved by the Ethical Commision of Airlangga University, but it did not involve human participants.

Competing Interests

All authors declare that they have no competing interests.

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Table 1. Anti-angiogenesis activity on the CAM model in each group

Treatment	% inhibition of neovascularization	% inhibition of endothelial cells
Celecoxib 60μg	83.3^{b} ±5.2	79.3^{c} ±2.3
FA 30μg	43.0^{a} ±4.6	42.6^{a} ±5.9
FA 60μg	83.4^{b} ±7.4	57.3^{b} ±4.3
FA 90μg	86.6^{b} ±6.2	70.7^{c} ±3.9

All values are represented as the mean ± SE (n=6) and different superscripts in the same column indicate that there were significant different in each group (P<0.05).

Table 2. Inhibition of VEGF and COX-2 Expression on CAM model in each group

Treatment	% inhibition of VEGF expression	% inhibition of COX-2 expression
Celecoxib 60μg	72.9^{b} ±6.8	84.1^{c} ±4.2
FA 30μg	46.7^{a}±8.0	49.9^{a} ±6.3
FA 60μg	63.7^{b}±4.9	55.0^{a} ±4.6
FA 90μg	73.6^{b} ±5.3	66.8^{b} ±2.8

All values are represented as the mean ± SE (n=6) and different superscripts in the same column indicate that there were significant different in each treatment (P<0.05).

Table 3. The Rerank score, type of bonding interactions and amino acids involved in the interaction of ligands with VEGFR-2 (PDB ID 4ASD)

Compound	Rerank Score (kcal/mol)	Number of H-bonds	amino acid residue	Number of Steric interaction	amino acid residue
FA	-73.844	1	Glu885	6	Ala1050, Glu885, Leu 889, Val 848
Celecoxib	-94.557	2	Gln 847 Lys 868	24	Asn1033,Cys1045, Gln 847, Glu885, Lys868, Phe1047, Val848, Val 867, Val

VEGFR-2: vascular endothelial growth factor receptor

Table 4. The Rerank score, type of bonding interactions and amino acids involved in the interaction of ligands with TKR (PDB 1XKK)

Compound	Rerank Score (kcal/mol)	Number of H-bonds	amino acid residue	Number of Steric interaction	amino acid residue
FA	-84.954	1	Lys745	9	Ala743, Gly796, Leu792, Leu844, Lys745, Met793, Val 726
Celecoxib	-93.163	2	Met793	24	Ala743, Asp855, Gln791, Leu792, Leu844, Lys745, Met793

TKR: tyrosine kinase receptor

Table 5.The Rerank score, type of bonding interactions and amino acids involved in the interaction of ligands with COX-2 (PDB 1CX2)

Compound	Rerank Score (kcal/mol)	Number of H-bonds	amino acid residue	Number of Steric interaction	amino acid residue
FA	-73.416	3	Ser353, His90	3	Tyr355,Val523, Leu352
Celecoxib	-118.107	5	Arg513 His90 Ser353 Leu352 Gln152	16	Tyr355,Val523, Val349, Ser530, Val116, Ser353, Leu352, Phe518

COX-2: cyclooxygenase-2