Antiangiogenesis activity test of tin leaf (Ficuscarica L.) on the number of blood vessels and VEGF expression of chorioallantoic membrane of embryonated chicken eggs

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Antiangiogenesis activity test of tin leaf *(Ficus carica L.)* on the number of blood vessels and VEGF expression of chorioallantoic membrane of embryonated chicken eggs

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Abstract

This research has been conducted on the antiangiogenesis activity test of the tin leaf (*Ficus carica L.*) on the number of blood vessels and vascular endothelial growth factor (VEGF) expression of chorioallantoic membrane (CAM) of embryonated chicken eggs. The general objective of this study is the use of natural products from the *F. carica L.* as chemopreventive in cancer through the mechanism of inhibition of angiogenesis. Inhibition of angiogenesis, supply of nutrients and oxygen to cancer cells will also be inhibited, so that indirectly cancer cells will be stunted. This study used 25 embryonated chicken eggs that divided into 5 treatments and each treatment has 5 repetitions. This treatment includes placebo negative controls, celecoxib-positive controls, and 3 treatment groups were given a water extract of *F. carica L.* which was divided into several doses of 75, 90, and 110 µg. The result found a significant difference ($P < .05$) in the macroscopic observation of the number of blood vessels in the CAM loaded with the paper dish. Observation of VEGF expression in vascular endothelial cells in the CAM also had a significant difference ($P < .05$). The optimal dose of water extract of *F. carica L.* of 90 µg has been able to inhibit the formation of new blood vessels by 65.51% and reduce VEGF expression by 45% in the chorioallantoic membrane. Thus, it can be concluded that the administration of water extract of *F. carica L.* can inhibit the formation of new blood vessels and inhibit VEGF expression.

Keywords: angiogenesis, chorioallantoic membrane, vascular endothelial growth factor expression

1. Introduction

The formation of new blood vessels, known as angiogenesis, is related to many pathological conditions such as diabetic retinopathy, atherosclerosis, tumor growth, and metastasis. Angiogenesis can be separated into several major steps, including: degradation of the basement membrane and extracellular matrix, migration, proliferation, and conversion of endothelial cells to form new blood vessels. Inhibition of angiogenesis has been considered beneficial for the prevention of neoplastic growth and chronic inflammatory diseases. Since angiogenesis plays a role in the development of several diseases, including tumor growth and metastasis, therefore, the discovery of antiangiogenic agents is very important. Latex from the *Ficus carica L.* is used in traditional Iranian medicine for the treatment of papillomatosis. In addition, it has been reported that latex extracts have different therapeutic effects such as hypoglycemic induction, cancer suppression, and anti-helminthic effects.[1]

Angiogenesis, the process of forming new vessels, is very important for the development and promotion of pathogenic processes of various disorders, including tumor growth and metastasis. Therefore, inhibition of angiogenesis can be useful in the treatment of tumors.[2]

Inhibition of angiogenesis is more effective than by killing the cancer cells directly in treating cancer. Inhibition of angiogenesis, supply of nutrients and oxygen to cancer cells will also be inhibited, so that indirectly cancer cells will be stunted.[3] Inhibition of angiogenesis can occur in the use of some drugs from plants or herbs, as in the test material that will be used in this study is *F. carica L.* Ethanol extract of *F. carica L.* contain antiangiogenic activity and can be a candidate as a potential agent for the prevention of disorders related to angiogenesis.[4]

One research model of angiogenesis uses the chorioallantoic membrane (CAM) of embryonated chicken eggs (ECEs). The CAM is often used in research on angiogenesis, tumor cell invasion, and metastasis. The CAM model has many advantages such as the enormous vascularity of CAM, allowing efficiency of tumor cell implantation, high reproducibility, simplicity and cost efficiency, and finally because CAM testing is a closed system, the half-life of several test compound molecules such as small peptides tends to be longer compared to animal models. Similarly, it allows experimental studies on the potential of anti-metastatic compounds that are only available in small quantities.

Based on observations that will be made, this study aims to prove the efficacy of *F. carica L.* leaf extract as antiangiogenesis

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on new blood vessels inhibition and vascular endothelial growth factor (VEGF) expression.

2. Materials and methods

2.1. Production of water extract of F. carica L.

The *F. carica L.* are washed with running water, drained, dried in the sun's heat indirectly covered with dark cloth. After being dried, pollinated and sieved until *F. carica L.* leaf powder is obtained. Take as much as 5 g of powder and put into a beaker glass, then add with distilled water with a temperature of 28°C to a volume of 200 mL. This process is carried out in an incubator for 24 h. After that the mixture is filtered with Whatman no.4 so that a water extract is obtained. This extract is then dried with freeze dryer and stored in a refrigerator with a temperature of −10°C.

2.2. Induction of angiogenesis with basic fibroblast growth factor

Induction of new blood vessels growth on CAM was carried out by giving angiogenesis factor, namely, basic fibroblast growth factor (bFGF). Inductors as much as 25 µg was diluted by adding 500 µL of Tris-HCl 10 mM buffer, pH 7.5 in cold conditions, so that the stock concentration of bFGF was 50 ng/µL. This stock was taken as much as 25 µL then diluted with the addition of 1225 µL Tris-HCl 10 mM buffer, pH 7.5 so that the concentration of recombinant human basic fibroblast growth factor was 1 ng/µL. The dose of bFGF applied to eggs was 30 ng, with a volume of up to 30 µL from the bFGF stock with a concentration of 1 ng/µL.

2.3. Making test solvent

The water extract of *F. carica L.* was weighed as much as 10 mg, then dissolved in 2% dimethyl sulfoxide (DMSO) in sterile aquabidestilata to obtain stock with a concentration of 1 µg/µL. Furthermore, the test solvent was filtered with a 0.22 µm filter, put in sterile flacon and made a dose series. The dose inoculated on CAM consists of 3 doses of 75, 90, and 110 µg per egg. The preparation of this test solution was carried out in laminar air flow-hood aseptically.

2.4. Inhibitory power test for angiogenesis

Nine-days ECEs are marked on eggshells which include airspace limits, location of embryos, and areas to be made of rectangular holes (windows) measuring 1 cm^2 above the embryo. The location of the embryo is known by candling on the egg. Eggshells at the poles that contain air and shells above the embryo are purified with 70% alcohol. In both areas, then a small hole is made using a small drill. Air from the air space is aspirated with a rubber ball to the CAM attached to the loose egg membrane. This treatment is carried out with horizontal egg position, in a dark room and using binoculars so that the CAM and artificial air space formed above the embryo can be seen. The eggs are purified again and put in a flow-hood water laminar in a horizontal position with artificial air space located at the top.[6]

2.5. Implantation test solution into the egg

The embryo eggshell is cut with a saw (mini drill) to form a rectangular hole with an area of 1 cm^2. Through this hole the test solution is implanted into the CAM. Test subjects in the form of eggs were divided into 5 groups as follows: (C+) positive control group, 5 ECEs with inductors, namely, bFGF as much as 60 ng and Tris-HCl solvent. As well as celecoxib 20 µg; (C−) negative control group, 5 ECEs by giving 2% Tris-HCl and DMSO solvents; (T1) the treatment group 1, 5 ECEs with the administration of inductors, namely, bFGF as much as 60 ng and water extract of *F. carica L.* as much as 75 µg in 2% DMSO; (T2) the treatment group 2, 5 ECEs with the administration of inductors, namely, bFGF as much as 90 ng and water extract of *F. carica L.* as much as 90 µg in 2% DMSO; (T3) treatment group 3, 4 ECEs with inductors, namely, bFGF as much as 60 ng and water extract of *F. carica L.* as much as 110 µg in 2% DMSO.

After being treated, the eggs were incubated at 38°C and 60% relative humidity for 72 h.[6] After that the egg is opened by cutting the eggshell into 2 parts starting from the shell that is close to the air cavity. The contents of the egg are gently removed so that the CAM remains attached to the eggshell. After that, macroscopic counting is done in the area around the paper disc. The CAM-containing blood vessels are collected in formalin buffer. Furthermore, the preparation of immunohistochemical histology preparations was carried out.

Data analysis was performed after data obtained from observations on the number of new blood vessels formed macroscopically and VEGF expression were analyzed statistically by analysis of variance (ANOVA) test. Then, compare the differences in each treatment group using Duncan's multiple range test.

3. Result and discussion

3.1. Macroscopic observation results

Macroscopic observation of the number of new blood vessels formed around and on the paper disc implanted in the CAM of ECEs is carried out. These observations can be made after going through the antiangiogenesis test by administering water extracts of *F. carica L.* The preparation of ECEs was carried out after the administration of the test material on ECEs and through the incubation period for 72 h in the incubator temperature 38 to 38.5°C. Macroscopic observations and comparison between bFGF control groups without extracts and with treatment groups with extracts are shown in Figure 1.

The growth of new blood vessels in the positive control group bFGF seemed to surround the paper dish even more on the surface. This seems to be influenced by the activation of bFGF which was previously dropped on the surface of the paper dish as a trigger for the growth of new blood vessels. When compared with the extract treatment group, it will look different, where around the paper dish is rarely overgrown with blood vessels. It means *F. carica L.* water extract has activity in inhibiting the formation of new blood vessels.

The observation is continued by counting the number of new blood vessels or capillaries that are formed from the branching of large blood vessels. The calculation results of each treatment group were made the average of the 4 replications in each treatment group. The average number of new blood vessels can be seen in Table 1.

Data of the number of new blood vessel performed using Kolmogorov-Smirnov test to know the normality of data distribution. Furthermore, to find out the difference in the number of new blood vessels with the administration of *F. carica L.* extract, various doses were analyzed using F test, giving significant results ($P < .05$).

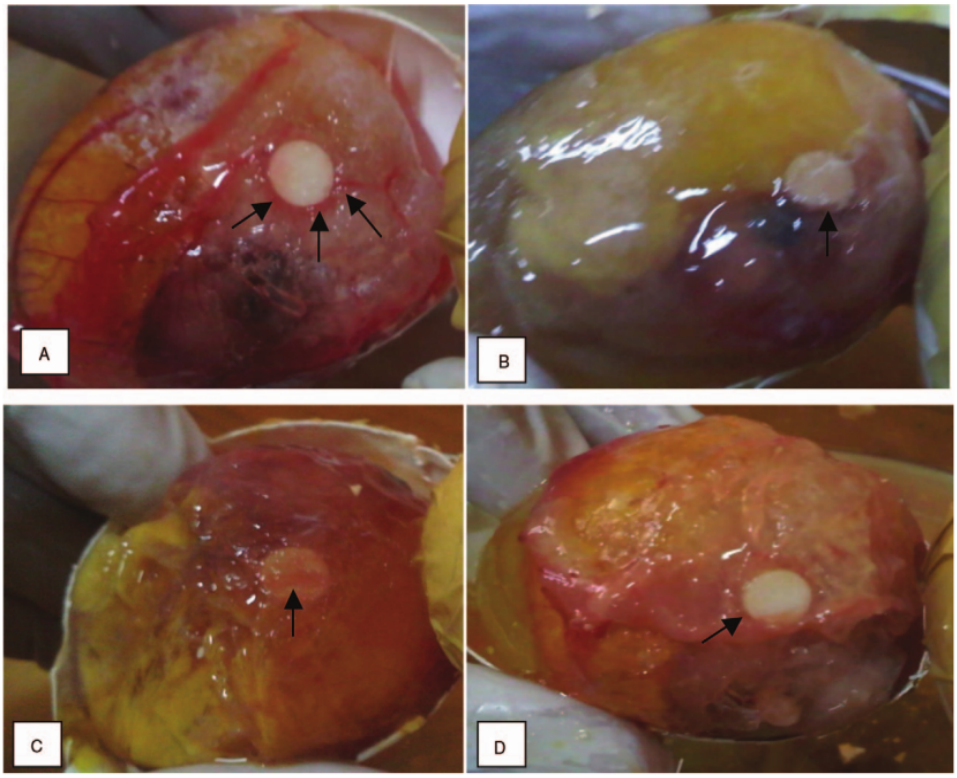

Figure 1. Appearance of blood vessel growth in the chorioallantoic membrane ECEs surface dish (arrow). It was a bFGF control group without extract (A), negative control (B), *Ficus carica L.* extract treatment group 750 μg (C) and 90 μg (D) treatment group. bFGF = basic fibroblast growth factor, ECE = embryonated chicken egg.

This shows that there are significant differences in the administration of *F. carica L.* extract with various doses to reduce the number of new blood vessels. The difference in the mean number of new blood vessels between treatments can be explained through further testing using Duncan's multiple range test. The lowest average number of new blood vessels was found in the treatment group of *F. carica L.* extract 110 μg + bFGF 60 ng at $2.20^{a} \pm 1.80$. These results were not significantly different ($P > .05$) with the positive control group celecoxib 20 μg of $3.40^{ab} \pm 1.30$ and the treatment group of *F. carica L.* extract 90 μg + bFGF 60 ng which was $3.80^{ab} \pm 130$. It means extracting *F. carica L.* for 110 μg was able to inhibit the growth of new blood vessels by the effect of 60 ng bFGF which was equivalent to the positive control group using the celecoxib drug, whereas in the treatment group with the administration of *F. Carica L.* extract 75 and 90 μg gave results that were not significantly different ($P > .05$), but these results indicate that there were obstacles to new blood vessels growth in the 3 treatment groups ($5.00^{bc} \pm 1.00$) and ($3.80^{ab} \pm 1.30$) when compared with the bFGF control group 60 ng ($5.80^{c} \pm 1.48$). The average number of new blood vessels in the positive control group administration of 20 μg of celecoxib synthetic drug gave a low yield of $3.40^{ab} \pm 1.30$, not significantly different ($P > .05$) or comparable to the 110 μg extract group which was $2.20^{a} \pm 1.80$.

Table 1

The average number of new blood vessels in the chorioallantoic membrane of embryonated chicken eggs (ECEs) in each treatment group.

Treatment group	Number of new blood vessels (average ± SD)
Positive controls of bFGF 60 ng + celecoxib 20 μg	$3,40^{ab} \pm 1.30$
Ris solvent negative controls and DMSO 2% + bFGF 60 ng	$5,80^{c} \pm 1.48$
Treatment I *Ficus carica L.* extract 75 μg + bFGF 60 ng	$5,00^{bc} \pm 1.00$
Treatment II *Ficus carica L.* extract 90 μg + bFGF 60 ng	$3,80^{ab} \pm 1.30$
Treatment III *Ficus carica L.* extract 110 μg + bFGF 60 ng	$2,20^{a} \pm 1.80$

Description: different superscripts in the same column mean that there were significant differences in each treatment ($P < .05$).
bFGF = basic fibroblast growth factor, DMSO = dimethyl sulfoxide.

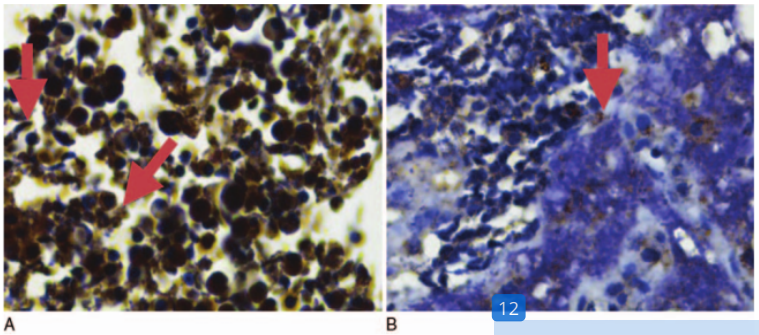

Figure 2. Observation of VEGF expression (arrows) with immunohistochemical methods. 400× magnification microscope. (A) shows the negative control group bFGF 60 ng and (B) shows the treatment group giving water extract of *Ficus carica L.* leaf 90 μg. bFGF = basic fibroblast growth factor, VEGF = vascular endothelial growth factor.

The results of the observation of the total number of new blood vessels in the treatment group giving *F. carica L.* extract showed less along with the addition of the extract dose. These results indicate that the higher the dose of *F. carica L.* extract, the higher the inhibition of new blood vessels growth.

3.2. VEGF expression observation results

VEGF expression in the cytoplasm and endothelial cell surface can be detected using anti-VEGF antibodies. The results of the calculation on the observation of VEGF expression in the control group were negative for placebo (giving bFGF 60 ng without extract). It appeared that endothelial cells scattered in the new blood vessels were dominated by the presence of brown color. The visualization results appear to be diminishing in the treatment group by giving water extract of *F. carica L.* leaves starting at doses of 75, 90, and 110 μg, as well as a positive control group compared with celecoxib 20 μg and the placebo negative control group as shown in Figure 2.

The results of the calculation of VEGF expression in each treatment group can be seen in Table 2.

Based on the results of statistical calculations of the number of VEGF expressions in the coronal endothelial membrane blood vessel endothelium, showed a significant difference ($P < .05$) in the treatment of the effect of water extracts of *F. carica L.* leaves while the optimal dose which had a significant difference was 75 μg. Even greater decreases in VEGF expression occurred in the administration of water extracts of *F. carica L.* leaves doses of 90 and 110 μg.

Based on the results of the study on the observation of number of new blood vessels formed in the paper dish loaded with bFGF, the observations of VEGF expression appeared to be linear, in which the water extract of *F. carica L.* decreased the number of new blood vessels formation with a decrease in the number of cells endothelium that expresses VEGF. Inhibition of angiogenic factor endogenous such as bFGF, VEGF, circulating endothelial progenitor cells, inhibits degradation enzymes (matrix metalloproteinases) which are responsible for the degradation of basal membrane, inhibits endothelial cell proliferation, inhibits endothelial cell transfer, and inhibits activation and differences of endothelial cells. bFGF and VEGF are the main components in the process of angiogenesis in wounds as well as in cancer cells.[7]

VEGF plays an important role in the regulation of angiogenesis and is the only endogenous-specific mitogen. Signal pathways mediated by VEGF in endothelial cells will determine the target of angiogenesis in blood vessels. Three main pathways in VEGF signals have been reported, which include signal-regulated mitogen-activated or extracellular kinase, mitogen-activated protein kinase pathway, phosphatidylinositol 3-kinase pathway, and focal adhesion kinase (Src-FAK) pathway. Peroxisome proliferator-activated receptor gamma coactivator-1 alpha transcription coactivator (peroxisome-proliferator-activated receptor-gamma coactivator-1 alpha), a potent sensor and metabolic regulator, induced by a lack of nutrients and oxygen.[8] Obstacles to VEGF by a compound mean that there are obstacles to the 3 main signaling pathways in endothelial cells for angiogenesis.[7]

The water extract of *F. carica L.* seems to play an important role in the inhibition of VEGF expression as well as the formation of new blood vessels. Methanol extract of *F. carica L.* leaves for inhibition of angiogenesis proves the occurrence of inflammatory

Table 2

Average number of endothelial cells expressing VEGF in each treatment after administration of *Ficus carica L.* leaf water extract and bFGF induction.

Treatment group	Number of VEGF expression (average ± SD)
Positive control of bFGF 60 ng + celecoxib 20 μg	$12,92^{a} \pm 1.29$
Ris solvent negative controls and DMSO 2% + bFGF 60 ng	$24,44^{c} \pm 3.27$
Treatment I *Ficus carica L.* extract 75 μg + bFGF 60 ng	$20,56^{b} \pm 2.29$
Treatment II *Ficus carica L.* extract 90 μg + bFGF 60 ng	$11,00^{a} \pm 1.72$
Treatment III *Ficus carica L.* extract 110 μg + bFGF 60 ng	$11,56^{a} \pm 2.76$

Description: different superscripts in the same column mean that there were significant differences in each treatment ($P < .05$).
bFGF = basic fibroblast growth factor, DMSO = dimethyl sulfoxide, VEGF = vascular endothelial growth factor.

barriers in rheumatoid arthritis in carrageenan-induced rats.[9] Similarly, the extract of doses of 5, and 50 mg/pouch significantly decreased the production of tumor necrotic factor α, prostaglandin F2, and VEGF so that there were obstacles to the parameters of angiogenesis and inflammation.[10] The antioxidative activities of water extract *F. carica L.* fruit were investigated using various assays in vitro, including scavenging abilities on 2, 2-diphenyl-1-picrylhydrazyl, crude hot water-soluble polysaccharide, superoxide, and hydroxyl radicals. The polysaccharide showed higher activity in water extract *F. carica L.* in the amount of 0.95 mg/mL.[11]

The administration of ethanol extract of *F. carica L.* significantly decreased the mRNA expression level of VEGF-A and β3 integrin in human umbilical vein endothelial cells (HUVECs) at 20 μg concentration of extract concentration compared with untreated control cells ($P < .05$). The extract dose directly inhibits the formation of tubes of HUVECs. The reduced level of mRNA expression from VEGF indicates an inhibition of VEGF-A expression and further inhibits the formation of new blood vessels.[4]

There are 2 classes of angiogenesis inhibitors, either directly or indirectly. Direct angiogenesis inhibitors such as vitaxin, angiostatin, and others, prevent vascular endothelial cell proliferation, migration, or prevent cell death due to the response of proangiogenic proteins, including VEGF, bFGF, interleukin-8, platelet-derived growth factor, and platelet-derived endothelial growth factor. Direct angiogenesis inhibitors are very weak in inducing drug resistance, because genetic targets for endothelial cells are stable compared to tumor cells that are unstable mutations.[12] The administration of latex extract of *F. carica L.* at a dose of 100 to 400 μg/mL showed an inhibition of endothelial cell proliferation that inhibits the formation of vascular capillary tubes in the HUVECs.[1]

4. Conclusion

It can be concluded that the administration of water extract of *F. carica L.* leaves at 90 to 110 μg can inhibit the formation of new blood vessels and inhibit VEGF expression of CAM of ECEs.

Conflicts of interest statement

There are no conflicts of interest to disclose.

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