# Antiangiogenic Effect of Ficus Carica Linn Leaf Extract on Total Blood Vessels, Matrix Metalloproteinase-9 Expression and Macrophages in the Chick Embryo Chorioallantoic Membrane

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# Antiangiogenic Effect of *Ficus Carica Linn* Leaf Extract on Total Blood Vessels, Matrix Metalloproteinase-9 Expression and Macrophages in the Chick Embryo Chorioallantoic Membrane

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**ABSTRACT**

**Background and Aim:** Inhibiting angiogenesis is suppose to be more effective in preventing potential cell metastases. Antiangiogenic substances inhibit the supply of nutrients and oxygen to cancer cells so that the development of protooncogens can be inhibited. This study aimed to evaluate the effect of *Ficus carica Linn* leaf extract as antiangiogenic on total blood vessels, matrix metalloproteinase-9 (MMP-9) expression and total macrophages on chorioalantoic membranes of chicken embryos.

**Materials and Methods:** A total of 25 chicken embryos were randomly assigned to 5 treatment groups, i.e. (C-) induced by Tris-HCl and 2% DMSO solvent; (C +) induced 60 ng of bFGF and Tris-HCl solvent; (T1) induced 60 ng of bFGF and 20 µg of celecoxib; (T2) induced 90 ng of bFGF and 90 µg of *F. carica L.* extract in 2% DMSO; (T3) was induced by 60 ng of bFGF and 110 µg of *F. carica L.* leaf extract in 2% DMSO. The total blood vessels observed macroscopically were counted on the main blood vessel branches around the paper disc. Total macrophages were observed on HE staining and MMP-9 expression on IHC staining. Data were analyzed by ANOVA test followed by Tukey's test ($p < 0.05$).

**Results:** The results show that T3 was significant ($p<0.05$) compared to C+ and T2 for all observed parameters. Meanwhile, T3 was not significant ($p>0.05$) compared to C- and T1 for all observed parameters.

**Conclusion:** It can be concluded that *F. carica L.* leaf extract at a dose of 110 µg in DMSO 2% can significantly reduce blood vessel total, MMP-9 expression and total macrophages in chicken embryos induced by bFGF. On the other hand, there was no significant difference between *F. carica L.* leaf extract at a dose of 110 µg in DMSO 2% with celecoxib treatment, which means that it gave the same effectiveness in reducing the effect of bFGF.

**Keywords:** Antiangiogenic, Ficus carica Linn, macrophage, matrix metalloproteinase

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## INTRODUCTION

Angiogenesis is a physiological process that involves the growth of new blood vessels [1]. In the process of cancer growth, angiogenesis acts in supplying nutrients and oxygen to cancer cells, carrying metabolic wastes and biological end products from cancer cells. Thus accelerating the spread of cancer cells to surrounding cells and tissues. The formation of angiogenesis indicates a change in the status of cancer cells, from dormant to malignant [2]. Cancer cells induce angiogenesis by secreting various growth factors such as Vascular Endothelial Growth Factor (VEGF) and Basic fibroblast growth factor (bFGF) [3].

Matrix metalloproteinase-9 (MMP-9) is a specific marker of heterophiles to indicate that angiogenesis initiates and expands [4]. Macrophages are the innate body defense system present in birds and function as phagocytosis. Innate body defenses are non-immunological defenses that have existed since the individual was born [5]. Cancer treatment through inhibition of angiogenesis is more effective in treating cancer than by directly killing the cancer cells [6]. Inhibition of angiogenesis, the supply of nutrients and oxygen to cancer cells will also be inhibited, so that cancer cells will indirectly be stunted [7]. Angiogenesis inhibition can occur in the use of several drugs of plant or herbal origin, such as the test material to be used in this study, namely the leaves of the Tin plant (*Ficus carica Linn*) [8]. The ethanol extract of the leaves leaving *F. carica L.* contains anti-angiogenic activity and could be a candidate as a potential agent for the prevention of angiogenesis-related disorders [9].

A highly representative angiogenesis research model using observation of blood vessels growing on the chorioalantois membrane (CAM) of 8 day old chicken embryos [10]. Vascular growth is easily observed and sensitive to growth factor inductors such as bFGF. Based on the observations to be carried out, this study aims to evaluate the efficacy of *F. carica L.* leaf extract as antiangiogenesis in total blood vessels, MMP-9 expression and total macrophages.

## MATERIALS AND METHODS

### Ethical approval

This study was approved by the Animal Ethics and Care Committee Faculty of Veterinary Medicine, Universitas Airlangga. The use of chicken embryos was considered as part of avoiding animal abuse

### Experimental design

The leaves of *F. carica L.* were taken randomly with fresh conditions then processed to obtain water extract [11]. Extract water using doses of 75, 90 and 110 µg. As an angiogenesis inductor, recombinant human bFGF (Nako Pure Chemical Industries Ltd. Japan) was used in buffering with Tris-HCl pH 7.5. The chemicals used include materials for extraction, namely Dimethylsulfoxide (DMSO) as an extract solvent, and 96% ethanol. In addition, 0.01 M Tris-HCl buffer solution with a pH of 7.5 was used as a bFGF solvent, 10% formalin to preserve CAM for histological

preparations, and hematoxillin and eosin as dyes for histological preparations [12].

A total of 25 chicken embryos were randomly assigned to 5 treatment groups, i.e. (C-) induced by Tris-HCl and 2% DMSO solvent; (C +) induced 60 ng of bFGF and Tris-HCl solvent; (T1) induced 60 ng of bFGF and 20 µg of celecoxib; (T2) induced 90 ng of bFGF and 90 µg of *F. carica L.* extract in 2% DMSO; (T3) was induced by 60 ng of bFGF and 110 µg of *F. carica L.* leaf extract in 2% DMSO. The total blood vessels observed macroscopically were counted on the main blood vessel branches around the paper disc.

**IHC and HE Staining**

The CAM was stored in formaldehyde 15% for 48 h. Furthermore, alcohol was used as a dehydration agent with concentration of 70%, 80%, and 96%. Xylol was used for clearing process and continued making paraffin block with 60°C of the temperature. The CAM that has received paraffin blocks then sliced using a microtome machine and then transferred into a water bath before being placed on a glass object. Immunohistochemistry staining was used primary antibody MMP-9 anti-mice for 1 h in 27°C. The dilution was given 10 µl for MMP-9. Then, the specimen washed in phosphate buffered saline (PBS) with a pH of 7.4 for 3 times every 5 min. The next preparations were added streptavidin-horseradish peroxidase for 60 min in 27°C and washed in PBS with pH 7.4. Then, the specimens were added chromogen 3,3-Diaminobenzidine tetrahydrochloride for 20 min and washed with aquadest for 5 min [13].

HE staining was conducted according to routine protocols [14]. Briefly, after deparaffinization and rehydration, 5 µm longitudinal sections were stained with hematoxylin solution for 5 min followed by 5 dips in 1% acid ethanol (1% HCl in 70% ethanol) and then rinsed in distilled water. Then the sections were stained with eosin solution for 3 min and followed by dehydration with graded alcohol and clearing in xylene. The mounted slides were then examined and photographed using a Nikon E200 trinocular microscope (Tokyo, Japan).

**Statistical analysis**

Data were expressed as mean ± standard deviation (SD) and analyzed statistically using ANOVA test followed by Tukey test for comparison between groups. Differences were considered significant at $p<0.05$. The analysis performed using SPSS v25 (IBM, USA).

## RESULTS AND DISCUSSION

The results show that T3 was significant ($p<0.05$) compared to C+ and T2 for all observed parameters. Meanwhile, T3 was not significant ($p>0.05$) compared to C- and T1 for all observed parameters (Table-1). Group C + showed the highest number of blood vessels on the paper disc, both new blood vessels around the paper disc and on the paper disc. Whereas in the T2 and T3 groups, there was a slight formation of new blood vessels from the main blood vessels, this shows the inhibition of new blood vessel growth activity by F. carica L. leaf extract (Figure-1).

**Table-1.** Total blood vessels, expression of MMP-9 and macrophages at the end of treatment

| Treatment | Blood vessel | MMP-9 | Macrophages |
|---|---|---|---|
| C- | $1.2 \pm 0.84^{a}$ | $23.64 \pm 3.08^{ab}$ | $11.68 \pm 1.38^{a}$ |
| C+ | $4.8 \pm 0.84^{c}$ | $44.68 \pm 0.52^{d}$ | $20.08 \pm 1.17^{c}$ |
| T1 | $1.0 \pm 1.00^{a}$ | $22.52 \pm 2.19^{a}$ | $12.36 \pm 0.77^{a}$ |
| T2 | $3.4 \pm 1.14^{b}$ | $39.28 \pm 1.38^{c}$ | $16.36 \pm 1.20^{b}$ |
| T3 | $1.2 \pm 1.09^{a}$ | $25.84 \pm 1.59^{b}$ | $12.32 \pm 1.01^{a}$ |

Data are expressed as mean ± standard deviation. Different superscripts $^{a,b,c,d}$ in the same column showed a significant difference ($p<0.05$).

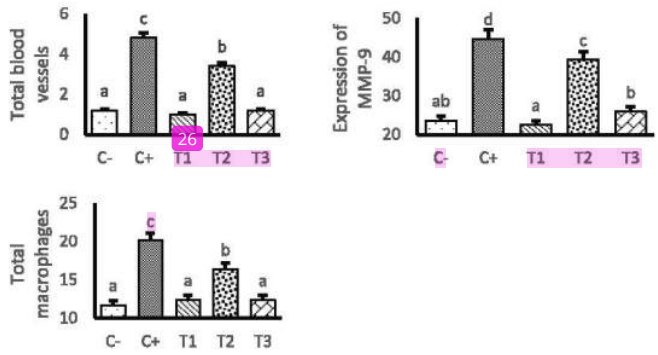



**Figure-1.** Total blood vessels, expression of MMP-9 and macrophages at the end of treatment. Data were expressed as mean ± 95% CI. Different superscripts $^{a,b,c,d}$ showed significant differences ($p<0.05$).

Angiogenesis is the formation of new blood vessels that can occur in both physiological and pathological conditions. The process of physiological angiogenesis begins in the womb and continues into old age. Vascularity is required in all tissues to ensure the availability of oxygen and nutrients [15]. Oxygen plays an important role in vascular regulation where hemodynamic factors are very important for the survival of vascular tissue and the

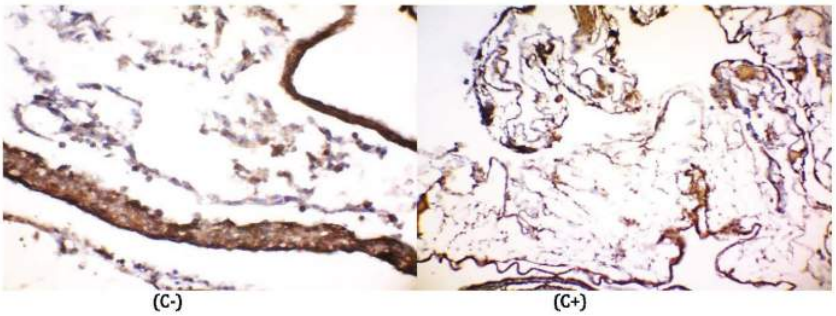
ptation of the structure of the vessel walls. Alterations in metabolic activity lead to proportional changes in angiogenesis and capillary changes [16].

Angiogenesis has long attracted attention for its role in health as well as disease. Angiogenesis is observed in more detail in the formation of new blood vessels in areas of injury or inflammation [17]. Abnormal characteristics of the blood vessels around the tumor. The study of tumor angiogenesis is much more advanced which makes it possible to study the morphological characteristics of blood vessel development in live animals [18].

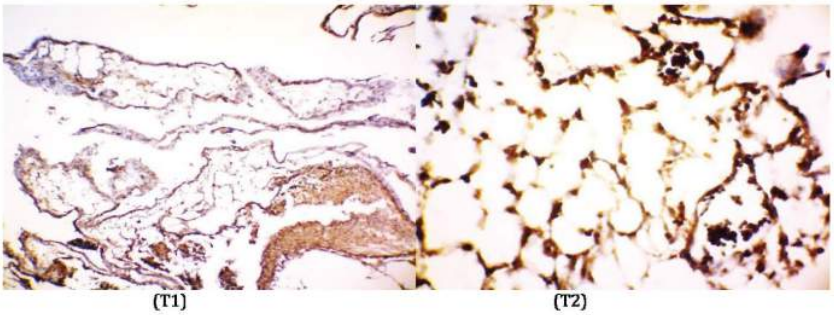
Tumor progression and metastasis are dependent on angiogenesis, and it is proposed that inhibition of giogenesis in tumors may be a strategy for inhibiting cancer growth and metastasis [19]. Angiogenesis in cancer is the process of forming new blood vessels from local blood vessels around the primary tumor rough a sprouting mechanism. Angiogenesis allows cancer cell owth and metastasis through the availability of nutrients and oxygen to cancer cells and surrou ng tissues, and also as a means of metastasis through which cancer cells can escape from the primary tumor and through the blood flow of angiogenesis, tumor cells will metastasize to a more distant location [20]. In general, the mechanism of angiogenesis in cancer is very complex, based on the influence of proangiogenic factors starting from the cess of endothelial proliferation and migration, growth factor signaling, extracellular matrix remodeling and stromal cell interactions [21].

Furthermore, it has been shown that the angiogenetic density in cancer is also important in predicting prognosis as well as cancer response to chemotherapy or radiotherapy [22]. However, perhaps the most important clinical implication of angiogenesis in cancer is the development of anti-angiogenesis therapies, ie therapies that target inhibit the mation of new vascularization around the tumor [23]. Vascular endothelial growth factor (VEGF) is a multifunctional cellular factor that can induce the formation of new blood vessels, increase capillary permeability through its direct effect ndothelial cells, stimulate endothelial mitosis and play an important role in the formation of new vascular systems and e growth of cancer cells. VEGF and its receptors have been shown to play an important role in the process of angiogenesis, not only in physiological conditions but also in angiogenesis in hological conditions, eg cancer [24].

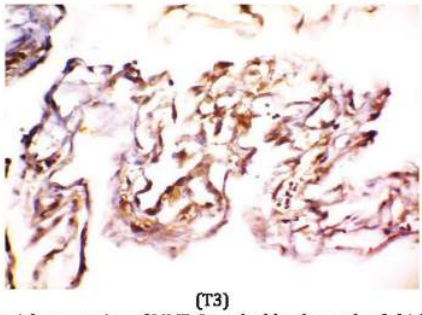
The results of our study using the predicted immunohistochemical method proved that VEGF protein expr on was increased in angiogenesis in the tumor area, in cancer cells and in the stromal cells a nd cancer. Endoglin (CD105) is a cell adhesion molecule expressed on the cell surface with the gene location at 9q34 a 180 kDa homodimeric transmembrane glycoprotein, a component of the TGF-β (Transforming Growth Factor-Beta) receptor complex which is a eotropic cytokine that modulates angiogenesis via 3 regulation of cell function including proliferation, differentiation and migration [25].



(C-) (C+)



(T1) (T2)



(T3)

**Figure-2.** Brownish expression of MMP-9 on the blood vessels of chick embryo CAM.

Using immunohistochemical methods, our study has also proven that the CD105 protein is exclusively expressed on angiogenetic endothelial cells in the tumor region assessed by its positive expression on endothelial cells. In contrast, CD105 protein is hardly expressed on capillary endothelial cells of normal tissue around the tumor [26]. The mechanism of blood vessel formation in physiological conditions which includes vasculogenesis and angiogenesis in cancer as well as immunohistochemical evaluation through the expression of VEGF protein, CD105 protein (Endoglin) and several angiogenic markers such as matrix metalloprotein-9 (MMP-9) (Figure-2) [27].

Macrophages are widely distributed in the human body. Macrophages play a role in the inflammatory process as the body's reaction to foreign objects or microbes. In neoplastic growth, macrophages are found in the extracellular space. Macrophages located in the extracellular space are known as tumor-associated macrophages (TAMs). It has been known for many years that TAMs are the main cellular component of cancer in humans. However, it is still difficult to understand how the process and mechanism are [28].

The role of macrophages is not only limited to the phagocytosis of foreign objects that enter the body. However, macrophages are found to be the key to fibrosis and angiogenesis. Fibrosis is important for tissue to recover and survive the external environment. Angiogenesis is also important because without new blood vessels nutrients cannot be obtained by the tissue so that the tissue will experience death. Furthermore, it appears that macrophages play a role in the development and metastasis of cancer cells through the induction of fibrosis and angiogenesis [29]. Therefore, it is important to find out more about the mechanism involved. Macrophages phagocytose pathogens, dead cells and some components in the extracellular matrix. Macrophages also function to regulate organ homeostasis and remodeling. During tissue regeneration or healing, macrophages stimulate angiogenesis and facilitate tissue remodeling by secreting proteases and growth factors. Macrophages in tissue can be identified by the expression of several markers, in humans the marker is CD68. The majority of macrophages are present in the perivascular area of the wound healing area [30].

Angiogenesis or neovascularization is the formation of new capillaries from existing blood vessels. Angiogenesis is an important component of inflammatory reactions and repair processes, which also occur during physiological growth and embryogenesis. Meanwhile, the formation of new blood vessels from angioblasts is referred to as vasculogenesis [31].

The formation of new blood vessels requires the growth of existing blood vessels and the fusion of these vessels, which is called a vascular anastomosis process. In the subventricular zone, macrophages gather to form the subventricular vascular plexus. Once this plexus is formed, the number of macrophages in the area will decrease. It is known that macrophages help in the formation of new blood vessels due to VEGF secretion which induces angiogenesis [32].

## CONCLUSION

In conclusion, *F. carica L.* leaf extract at a dose of 110 µg in DMSO 2% can significantly reduce blood vessel total, MMP-9 expression and total macrophages in chicken embryos induced by bFGF. On the other hand, there was no significant difference between *F. carica L.* leaf extract at a dose of 110 µg in DMSO 2% with celecoxib treatment, which means that it gave the same effectiveness in reducing the effect of bFGF.

**Authors' Contributions**

ISH supervised the study. FF and ISH conducted the study. MTEP helped in the statistical analysis of the data. MTEP and FF helped in the preparation of tables, revised, and submitted the manuscript. All authors read and approved the final manuscript.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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