RESEARCH ARTICLE

Editorial Process: Submission:03/16/2022 Acceptance:01/08/2023

Antiangiogenic Activity of n-hexane Insoluble Fraction and Its Tylophorine Component from *Ficus septica* Leaves in Chicken Chorioallantoic Membrane Induced by bFGF

Devika Nurhasanah[1,2], Nanang Fakhrudin[3,4]*, Bambang Retnoaji[5], Agung Endro Nugroho[6]

Abstract

Objective: *Ficus septica* is an Indonesian medicinal plant traditionally used to treat various illness, including cancer. The n-hexane insoluble fraction of the ethanolic extract of *F. septica* leaves (HIFFS) shows a potential anticancer activity against breast cancer cell line T47D. Considering that angiogenesis is a pivotal factor in malignant cancer growth, progression, and invasion, we aimed to investigate the antiangiogenic effect of HIFFS on chicken chorioallantoic membrane (CAM) induced by bFGF. We also evaluated tylophorine, the cytotoxic alkaloid of *F. septica*. **Methods:** Chicken CAM was used to assess the antiangiogenic effect. Fertilized chicken eggs were induced with basic fibroblast growth factor (bFGF) ex ovo. Prior to bFGF induction, HIFFS (2.33, 4.65, 6.98, and 9.30 μg/mL) or tylophorine (9.20 μM) was added (10 μL) to a paper disk and implanted to the CAM. After 48 h of incubation, each treatment group was photographed, and the number of new blood vessel was calculated and compared with that in the solvent-treated group to determine the antiangiogenic activity. Histology of the CAM was evaluated after hematoxylin–eosin and Mallory acid fuchsin staining. **Results:** We found that HIFFS at low concentrations (2.33, 4.65, 6.98, and 9.30 μg/mL) inhibited angiogenesis activity (31.87, 41.99, 53.65, and 70.08, respectively) in chicken CAM induced by bFGF. Tylophorine (9.20 μM) also showed similar antiangiogenesis activity in the same model. Histopathology analysis revealed that HIFFS and tylophorine reduced the number of new blood vessels in CAM induced by bFGF. **Conclusion:** HIFFS and tylophorine showed antiangiogenic effect on chicken CAM induced by bFGF. This finding emphasized the potential of *F. septica* as a candidate anticancer agent.

Keywords: Anticancer- alkaloid- CAM- histopathology- angiogenesis

Asian Pac J Cancer Prev, **24 (1)**, 75-80

Introduction

Angiogenesis is the natural process of forming new blood vessels from existing blood vessels and plays a crucial role in wound healing and reproduction. Although considered as a normal bodily process, angiogenesis is also required by cancer cells to grow and develop. Its presence indicates that the cancer cells have extensive grown or metastasized to other tissues. Angiogenesis is needed by solid cancer cells for their metastasis and feeding through the blood flow in newly formed vessels. Angiogenesis inhibition is one of therapeutic approaches in combating cancer. The number of invasive cancer cells is proportional with that of blood vessels. When the formation of new blood vessels is inhibited, the access of malignant cancer cells to nutrition and oxygen is hindered (Potente et al., 2011; Eelen et al., 2020; Lugano et al., 2020), and the invasive cancer cells are consequently killed.

Angiogenesis is induced by various proangiogenic factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). During cancer cell progression, VEGF increases endothelial cell proliferation and migration, and bFGF induces cancer cells to stimulate new blood vessel formation and increases tumor volume (Murakami and Simons, 2008; Matkar et al., 2017). In this study, we used the chicken angiogenic experimental model of chorioallantoic membrane (CAM) induced by bFGF to mimic the angiogenesis of cancer cells. This method was adopted in previous studies to assess the antiangiogenic activity of anticancer agents

*[1]Postgraduate Program of Pharmaceutical Sciences of Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. [2]Department of Pharmacy, Faculty of Health, Universitas Jenderal Achmad Yani Yogyakarta, Yogyakarta,Indonesia. [3]Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. [4]Medicinal Plants and Natural Products Research Center, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia. [5]Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia. [6]Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. *For Correspondence: nanangf@ugm.ac.id*

(Ribatti, 2008; Au Dohle et al., 2009).

To date, angiogenesis inhibition remains one of the promising therapeutic targets of effective anticancer agents (Marmé, 2018; Teleanu et al., 2019; Eelen et al., 2020; Saman et al., 2020) and in angiogenesis-dependent diseases, such as degenerative diseases and diabetic retinopathy (Fallah et al., 2019). Medicinal plants have inspired the discovery of many bioactive molecules, including compounds for cancer treatment. The development of anticancer drugs from medicinal plants has attracted researchers worldwide. Current effective anticancer drugs, such as Taxol and vincristine, are originally derived from medicinal plants (Iqbal et al., 2017). Indonesia is a country with high biodiversity and therefore has diverse species of medicinal plants.

One of the Indonesian medicinal plants being studied for anticancer is *Ficus septica* (locally called "Awar-awar"). Previous studies showed that the ethanolic extract of *F. septica* leaves showed anticancer activity in several cancer cell lines (Septhea et al., 2011; Nugroho et al., 2015; Sutejo et al., 2016). Furthermore, the n-hexane-insoluble fraction of the ethanolic extract of *F. septica* leaves (HIFFS) exhibited a potential cytotoxic activity on T47D cell line (Nugroho et al., 2011). However, no data are available regarding the antiangiogenic activity of this fraction. HIFFS is an alkaloid fraction of *F. septica* leaves and contains tylophorine as its cytotoxic compound (Nugroho et al., 2015; Pratama et al., 2018; Muhammad Hafizh et al., 2020). In this study, we evaluated the antiangiogenic activity of HIFFS and its cytotoxic active compound, tylophorine, in an ex ovo culture of chicken CAM.

Materials and Methods

Plant material

F. septica leaves were obtained from Sumber Arum village, Moyudan District, Sleman, Yogyakarta, Indonesia, and the plant was verified by a botanist Dr. Djoko Santosa from the department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada (number: BF/281/Ident/Det/VI/2014). Only mature leaves were used for the experiment, washed using flowing water, cut into small sizes, and left at room temperature for 1 day before being dried in an oven (50°C for 48 h) on the next day. The dried plant material was powdered and used for fraction preparation.

Chemicals

The materials used for the experiment were ethanol (BrataChem, Yogyakarta, Indonesia), tylophorine (TRC Canada; Cat. Number T898200), bFGF (Sigma Aldrich, Cat. Number F5392), phosphomolybdic acid and albumin (Sigma Aldrich), phosphate buffer saline, polyethylene glycol, NaCl, toluene, xylol, hematoxylin, eosin, acid fuchsin, and albumin (Merck), and fertile chicken eggs (Maguwoharjo lokal Farm, Yogyakarta).

Fraction preparation

The dried powder of *F. septica* leaves was macerated in ethanol–water mixture (70:30) overnight. The residue was filtered, and the macerate was evaporated on a rotary evaporator (60 ˚C) and then stored in a desiccator until dried. The dried extract was fractionated using n-hexane to remove fats and chlorophylls to yield n-hexane soluble and insoluble fractions. The n-hexane insoluble fraction of the ethanol extract of *F. septica* leaves (HIFFS) was evaporated on a rotary evaporator to obtain the dried fraction for the experiment.

HPLC analysis

HIFFS was subjected to HPLC analysis using HPLC instrument Shimadzu (LC 6) with the following conditions: column, Symmetry Shield RP-18 (4.6 × 150 nm); mobile phase, 75 metanol-25 diethylamine 0.1%; pressure, 300~400 psi; flowrate, 1.0 mL/minute; running time, 25 minutes; and detection, UV at 275 nm.

Antiangiogenic assay

The method was approved by the institution ethic committee of the Laboratory of Integrative Research in the University of Gadjah Mada, Indonesia (certificate number: 00151/04/LPPT/XII/2017). Antiangiogenic activity was evaluated according to the ability to inhibit the formation of blood vessels necessary for the growth of chicken embryos. This study has also followed AIMRDA-Standard-Reporting-for-Anticancer Activity of Natural-Componds-Tool (Ahmad et al., 2022). The antiangiogenic assay was performed ex ovo using fertile chicken (Gallus gallus domesticus) eggs as previously described (Saraswati et al., 2013a). The fertilized eggs were incubated for 48 h at 37°C–39°C to stimulate the formation of new blood vessels at the CAM. The embryo was then separated from the shell and further incubated for 4 days. Tylophorine (9.2 μM), HIFFS (2.33, 4.65, 6.98, and 9.30 μg/mL), or solvent (PBS containing PEG 400) at 10 μL was added to the small paper disc and then implanted in the CAM, which was then added with bFGF (30 ng/μL) and incubated for 48 h for angiogenesis induction. At the end of experiment, the CAMs were stained with hematoxylin–eosin and Mallory acid fuchsin to visualize the newly formed blood vessels and the surrounding mesenchymal tissues in the CAM, respectively. Microscopic and macroscopic observations were performed to assess the effectivity of the treatment. Antiangiogenesis activity was evaluated by observing the response of the main blood vessel and calculating the number of new blood vessels in the paper disc. All treatments were photograph and documented for data analysis. Histopathology analysis was conducted under a microscope.

Results

In this experiment, we prepared the HIFFS from the ethanolic extract of *F. septica* leaves using n-hexane fraction. The HPLC profile of the HIFFS was compared with that of tylophorine, the cytotoxic alkaloid in *F. septica* that exhibits antiangiogenic activity (Saraswati et al., 2013b). As shown in Figure 1, HPLC analysis revealed the presence of tylophorine in HIFFS. In the angiogenesis assay, CAM was successfully induced by bFGF (Nakamichi et al., 2016; Yamamoto et al., 2020).

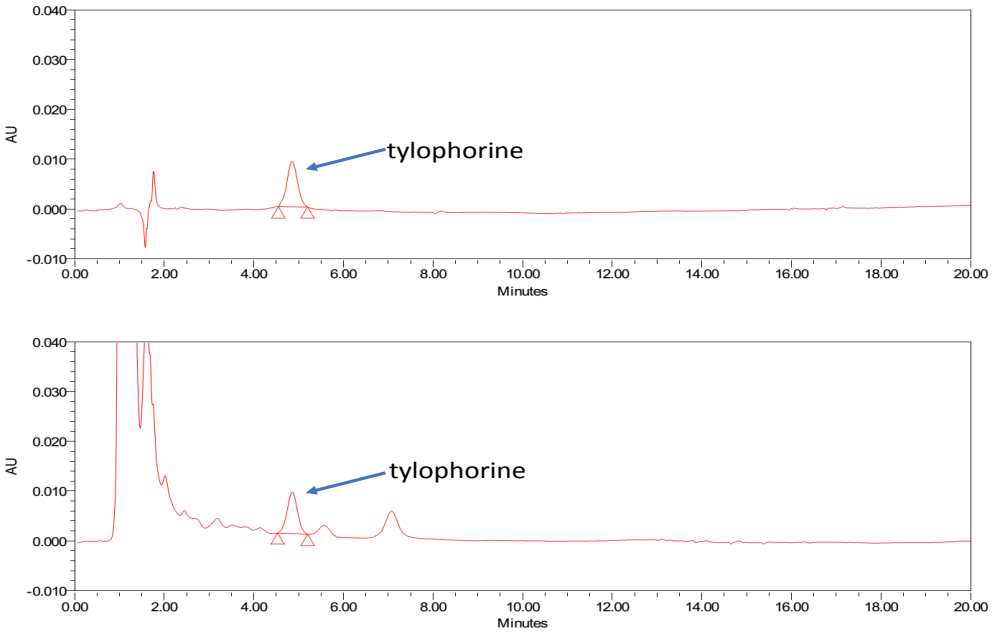

Figure 1. HPLC Profile of HIFFS and Tylophorine. A, HIFFS; B, Tylophorine. Stationary phase, Symmetry Shield RP-18 (reverse phase); mobile phase, methanol; flowrate, 1 mL/min; detector, UV 275 nm; run time, 15 min.

Macroscopic evaluation focusing on the area around the paper disk (Figure 2) showed that the new blood vessels (indicated by black arrows) were formed from the main blood vessels (indicated by blue light arrows). The new blood vessels showed a radial pattern centered on the implanted paper disk. This finding indicated that bFGF successfully induced angiogenesis in the experimental model. We found that the HIFFS and tylophorine groups had lower number of new blood vessels compared with the control group (Figures 2A–F), thereby suggested the antiangiogenic potential of these substances. To quantify the antiangiogenic effect, we counted the number of new blood vessels (indicated by black arrows around the implanted paper disk) in each group and presented the data in Figure 2. We found that HIFFS at 2.33, 4.65, 6.98, and 9.30 µg/mL significantly inhibited angiogenesis (31.87%, 41.99%, 53.65%, and 70.08%, respectively) in a dose-dependent manner (Figure 3). As expected, tylophorine (9.20 µM) potently inhibited the formation of new blood vessels with 87.26% or stronger inhibition than that of HIFFS at the highest concentration (9.30 µg/mL).

Histological analysis was performed microscopically

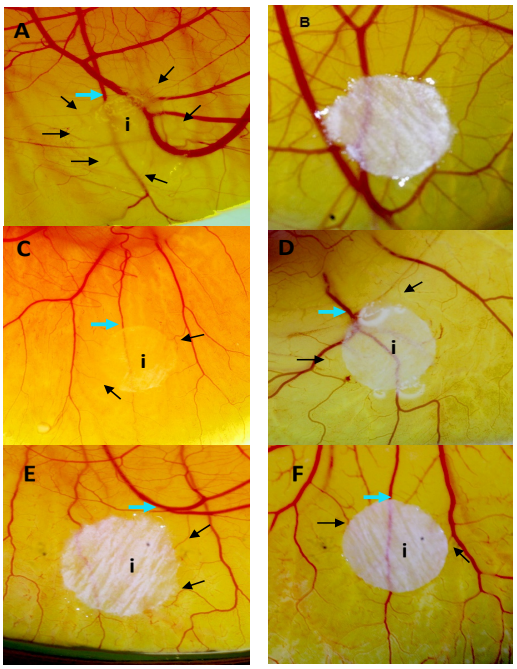

Figure 2. Representative Photographs Showing CAMs with the Old and New Blood Vessels. The images were taken from the ventral side on day 8 of incubation. A, solvent; B, Tylophorine 9.2 µM; C, D, E, and F were HIFFS at concentrations of 9.30, 6.98, 4.65, and 2.33 µg/mL, respectively. i, implanted paper disc; blue light arrow, main blood vessel; black arrows, new blood vessel.

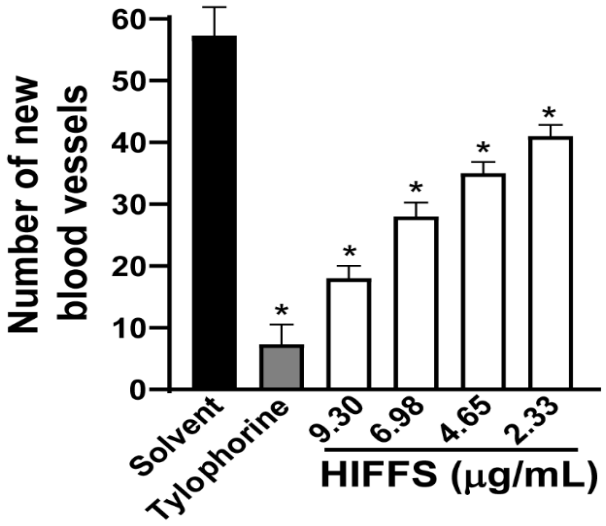

Figure 3. Antiangiogenic Activity of HIFFS and Tylophorine on Chicken CAM Induced by bFGF. The antiangiogenic activity was evaluated according to the ability to inhibit the formation of new blood vessels. The solvent was PBS containing PEG 400; tylophorine was tested at 9.2 µM; bFGF (30 ng/µL) was used to induce angiogenesis in the CAM. The data are means ± SD from three independent experiment (n = 3). *significant ($p > 0.05$) using one-way ANOVA, followed by LSD post hoc.

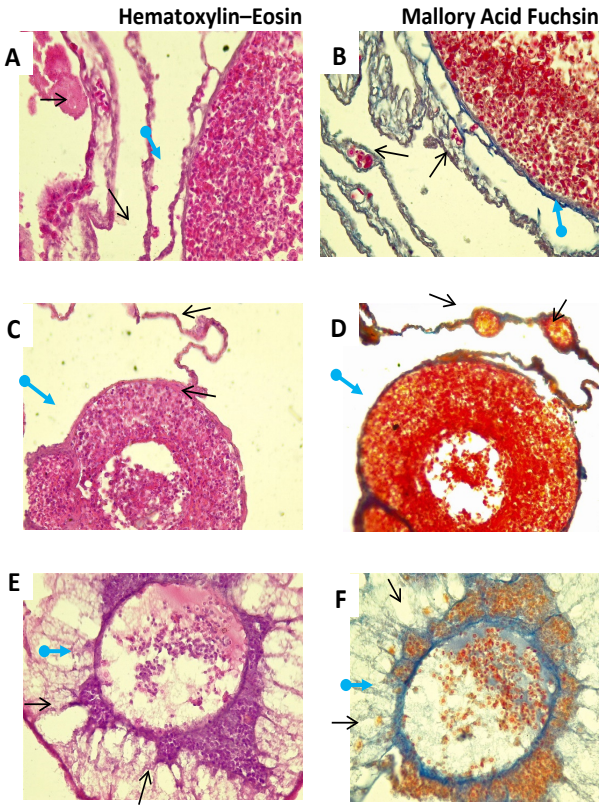

Figure 4. Representative Histological Cross-section Images of the CAM Containing Blood Vessels. The photographs were taken under a microscope on day 8 of incubation, and the CAMs were stained with hematoxylin–eosin (left panel), and Mallory acid fuchsin (right panel). A and B, solvent; C and D, Tylophorine (9.2 µM); E and F, HIFFS 9.30 µg/mL. Blue light arrow, main blood vessel; black arrow, new blood vessel; magnification, 40 × 10.

to confirm the antiangiogenic activities of HIFFS and tylophorine. Angiogenesis can be characterized by the thickness of blood vessel wall, the height density of erythrocytes in the vessels, and the thickness of mesenchymal tissue density. After hematoxylin–eosin staining, histology evaluation showed that the solvent-treated group (Figure 4A) had more thin-walled blood vessels (indicated by black arrows) than the HIFFS and tylophorine groups (Figures 4C and 4E). Thin walls are typical of new blood vessels formed through angiogenesis. This finding indicated that HIFFS and tylophorine inhibited angiogenesis. On the basis of histology evaluation after Mallory acid fuchsin staining, the density of the mesenchymal tissues in the solvent group was higher than that in the HIFFS and tylophorine groups (Figures 4B, 4D, and 4F). Given that the density of mesenchymal tissues is proportional to the number of new blood vessels (Mukhopadhyay et al., 1998; Yao et al., 2020), this finding reconfirmed the antiangiogenic activity of HIFFS and tylophorine.

Discussion

F. septica is a promising herbal medicine traditionally used to treat various diseases, including cancer (Lansky et al., 2008). This plant contains cytotoxic alkaloids, mainly phenantroindolizidine alkaloids, which have tylophorine as one of the main constituents (Wu et al., 2002). The extract and HIFFS exhibit cytotoxic activity and thus have potential to be developed as anticancer agents (Nugroho et al., 2011; Nugroho et al., 2013; Sutejo et al., 2016). In this study, we evaluated the antiangiogenic activity of HIFFS, an alkaloid-containing fraction of *F. septica* leaves, using a CAM model developed from chicken eggs. This method is relatively simple and effective for assessing the growth of blood vessels ex ovo (Au Dohle et al., 2009)

Angiogenesis is required by malignant cancer cells to survive and further develop. Although the anticancer activities of *F. septica* extract have been described, the antiangiogenic activity of its alkaloid-containing fraction is unknown. The antiangiogenic activity of tylophorine from Tylophora indica has been characterized in a different experimental model of sponge implant angiogenesis induced by VEGF (Saraswati et al., 2013b). Here we demonstrated additional evidence that tylophorine exerts antiangiogenic activity in chicken CAM induced by bFGF. These finding suggested that tylophorine might be the active compound responsible for the antiangiogenic activity of *F. septica*. However, the antiangiogenic activity of other compounds in *F. septica* must be further assessed to decipher the most potent compound. In addition to tylophorine, *F. septica* leaves also contain other cytotoxic phenantroindolizidine alkaloids with high structural similarities, such as ficuseptine, (+) -tylophorine, (+)-tylocrebrine, (+)-isotylocrebrine, antofin, and esculine (Wu et al., 2002).

Several cytotoxic aminocaprophenone- and pyrrolidine-type alkaloids have been identified in this plant (Ueda et al., 2009). From the pharmacological

point of view, the mechanism by which tylophorine and other alkaloids in *F. septica* inhibit angiogenesis must be elucidated. In this regard, thorough investigation must be conducted on tyrosine kinase receptor pathways that involve mitogen-activated protein kinases; among which, PI3k/Akt is the most relevant because bFGF activates these receptors (Loizzi et al., 2017).

Author Contribution Statement

AEN and NF formulated the idea of the manuscript; DN and NF wrote the initial draft; DN, NF, AEN and BR reviewed and edited the draft.

Acknowledgments

We thank Setiono and Lailiana Garna Nurhidayati for their technical assistance during the experiment. This study was supported by the Ministry of Research, Technology and Higher Education and Rispro Invitasi LPDP (Grant number KEP-85/LPDP/2019). This data was used by Devika Nurhasanah for her Master Thesis in the Faculty of Pharmacy, Universitas Gadjah Mada. This study was approved by the institution ethic committee of the Laboratory of Integrative Research in the University of Gadjah Mada, Indonesia.

Funding statement

This research was supported by the Ministry of Research, Technology and Higher Education, Republic of Indonesia and Rispro Invitasi LPDP, Ministry of Finance, Republic of Indonesia (Grant number KEP-85/LPDP/2019).

Conflict of interest

All authors declared no conflicts of interest.

References

Ahmad R, Riaz M, Aldholmi M, et al (2022). Development of a critical appraisal Tool (AIMRDA) for the peer-review of studies assessing the anticancer activity of natural products: A Step towards Reproducibility. *Asian Pac J Cancer Prev*, **22**, 3735-40.

Au Dohle DS, Au Pasa SD, Au Gustmann S, et al (2009). Chick ex ovo culture and ex ovo CAM assay: How it really works. JoVE, e1620.

Eelen G, Treps L, Li X, et al (2020). Basic and therapeutic aspects of angiogenesis updated. *Circ Res*, **127**, 310-29.

Fallah A, Sadeghinia A, Kahroba H, et al (2019). Therapeutic targeting of angiogenesis molecular pathways in angiogenesis-dependent diseases. *Biomed Pharmacother*, **110**, 775-85.

Iqbal J, Abbasi BA, Mahmood T, et al (2017). Plant-derived anticancer agents: A green anticancer approach. *Asian Pac J Trop Biomed*, **7**, 1129-50.

Lansky EP, Paavilainen HM, Pawlus AD, et al (2008). Ficus spp. (fig): Ethnobotany and potential as anticancer and anti-inflammatory agents. *J Ethnopharmacol*, **119**, 195-213.

Loizzi V, Del Vecchio V, Gargano G, et al (2017). Biological pathways involved in tumor angiogenesis and bevacizumab based anti-angiogenic therapy with special references to ovarian cancer. *Int J Mol Sci*, **18**, 1967.

Lugano R, Ramachandran M, Dimberg A (2020). Tumor angiogenesis: Causes, consequences, challenges, and opportunities. *Cell Mol Life Sci*, **77**, 1745-70.

Marmé D (2018). Tumor angiogenesis: A key target for cancer therapy. *Oncol Res Treat*, **41**, 164.

Matkar PN, Ariyagunarajah R, Leong-Poi H, et al (2017). Friends turned foes: Angiogenic growth factors beyond angiogenesis. *Biomolecules*, **7**, 74.

Muhammad Hafizh R, Andayana Puspitasari G, Subagus W, et al (2020). Application of ultrasonic-assisted fractionation with full-factorial design for the production of alkaloid-rich fraction from *Ficus septica* leaves. *J Food Pharm Sci*, **8**, 294-305.

Mukhopadhyay D, Tsiokas L, Sukhatme VP (1998). High cell density induces vascular endothelial growth factor expression via protein tyrosine phosphorylation. *Gene Expr*, **7**, 53-60.

Murakami M, Simons M (2008). Fibroblast growth factor regulation of neovascularization. *Curr Opin Hematol*, **15**, 215-20.

Nakamichi M, Akishima-Fukasawa Y, Fujisawa C, et al (2016). Basic fibroblast growth factor induces angiogenic properties of fibrocytes to stimulate vascular formation during wound healing. *Am J Pathol*, **186**, 3203-16.

Nugroho AE, Akbar FF, Wiyani A, et al (2015a). Cytotoxic effect and constituent profile of alkaloid fractions from ethanolic extract of *Ficus septica* Burm. F. leaves on T47D breast cancer cells. *Asian Pac J Cancer Prev*, **16**, 7337-42.

Nugroho AE, Hermawan A, Putri DD, et al (2013). Combinational effects of hexane insoluble fraction of *Ficus septica* Burm. F. and doxorubicin chemotherapy on T47D breast cancer cells. *Asian Pac J Trop Biomed*, **3**, 297-302.

Nugroho AE, Ikawati M, Hermawan A, et al (2011). Cytotoxic effect of ethanolic extract fractions of Indonesia plant *Ficus septica* Burm. F. on human breast cancer T47D cell lines. *Int J Phytomedicine*, **3**, 216-26.

Nurhidayati LG, Nugroho AE, Retnoaji B, et al (2021), Antiangiogenesis Activity of Awar-Awar Leaf Extract (Ficus septica Burm. f.) in Chorioallantoic Membrane Assay. *Indones J Pharm*, **32**, 1-9.

Pratama NP, Wulandari S, Nugroho AE, et al (2018). Tylophorine abrogates G2/M arrest induced by doxorubicine and promotes increased apoptosis in T47D breast cancer cells. *Asian Pac J Cancer Prev*, **19**, 3065-9.

Ribatti D (2008). Chapter 5 Chick embryo chorioallantoic membrane as a useful tool to study angiogenesis. In "International Review of Cell and Molecular Biology", Eds Academic Press, **270**, 181-224.

Saman H, Raza SS, Uddin S, et al (2020). Inducing angiogenesis, a key step in cancer vascularization, and treatment approaches. *Cancers*, **12**, 1172.

Saraswati S, Kanaujia PK, Kumar S, et al (2013a). Tylophorine, a phenanthraindolizidine alkaloid isolated from Tylophora indica exerts antiangiogenic and antitumor activity by targeting vascular endothelial growth factor receptor 2-mediated angiogenesis. *Mol Cancer*, **12**, 82.

Saraswati S, Kanaujia PK, Kumar S, et al (2013b). Tylophorine, a phenanthraindolizidine alkaloid isolated from Tylophora indica exerts antiangiogenic and antitumor activity by targeting vascular endothelial growth factor receptor 2–mediated angiogenesis. *Mol Cancer*, **12**, 82.

Septhea DB, Anindyajati A, Darma AP, et al (2011). *Ficus septica* Burm. F. leaves ethanolic extract induces apoptosis in 7,12-dimethylbenz[A]nthracene-induced rat liver cancer quatitavely. *IJCC*, **2**, 255-60.

Sutejo IR, Putri H, Meiyanto E (2016). Ethanolic leaves extract of Awar-awar (Ficus septica) as selective chemopreventive

agent on various cancer cells. *Nurs Line J*, **1**, 190-7.
Teleanu RI, Chircov C, Grumezescu AM, et al (2019). Tumor angiogenesis and anti-angiogenic strategies for cancert. *J Clin Med*, **9**, 84.
Ueda JY, Takagi M, Shin-Ya K (2009). Aminocaprophenone- and pyrrolidine-type alkaloids from the Leaves of Ficus septica. *J Nat Prod*, **72**, 2181-3.
Wu PL, Rao KV, Su CH, et al (2002). Phenanthroindolizidine alkaloids and their cytotoxicity from the leaves of Ficus septica. *Heterocycles*, **57**, 2401-8.
Yamamoto N, Oyaizu T, Enomoto M, et al (2020). VEGF and bFGF induction by nitric oxide is associated with hyperbaric oxygen-induced angiogenesis and muscle regeneration. *Sci Rep*, **10**, 2744.
Yao Z, Liu H, Yang M, et al (2020). Bone marrow mesenchymal stem cell-derived endothelial cells increase capillary density and accelerate angiogenesis in mouse hindlimb ischemia model. *Stem Cell Res Ther*, **11**, 221.