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ANTIANGIOGENESIS ACTIVITY OF AWAR-AWAR LEAF ETHANOLIC EXTRACT (*Ficus septica* Burm. f.) BY CHICK CHORIOALLANTOIC MEMBRANE METHOD

Lailiana Garna Nurhidayati¹, Agung Endro Nugroho², Bambang Retnoaji³, Sudarsono⁴, Nanang Fakhruadin⁴

¹Master Program, Faculty of Pharmacy. Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
²Department of Pharmacology and Clinical Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
³Faculty of Biology, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia
⁴Departement of Biology Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia

Email: lailianagarna@gmail.com

ABSTRACT

Cancer is one of the biggest causes of death after cardiovascular disease. Alkaloid content of *awar-awar* leaf extract (*Ficus septica* Burm. f.) is reported to exhibit cytotoxic activity in several cancer cells. This research aims to identify the antiangiogenesis potential of *awar-awar* leaf extract (*Ficus septica* Burm. f.) on chorioallantoic membrane (CAM) of chicken embryo induced with basic fibroblast growth factor (bFGF). *Awar-awar* leaf extract was obtained by the maceration method by using ethanol (70%), CAM was cultured in ex vivo and then angiogenesis induced by bFGF and treated by using the concentrated extract of 0.013; 0.00975; 0.0065; and 0.00325 mg/mL. The observation was done macroscopically and microscopically. Macroscopic observation result showed that an inhibition occurred at the concentration extract of 0.013 mg/mL or equal to 62.30%, 0.00975 mg/mL or equal to 51.37%, 0.0065 mg/mL or equal to 28.42%, and 0.00325 mg/mL or equal to 13.11%. At higher concentration of the extract, the growth of new blood vessels was decreased, demonstrated by the reduce erythrocyte density, parallel blood vessel pattern, and looser cellular density mesenchymal tissue. Moreover, the statistical analysis result showed that increasing concentration of extract could increase the inhibition of new blood vessels growth (p <0.05). So, *awar-awar* leaf ethanol extract can be used as an antiangiogenesis agent in CAM of chicken embryo induced with bFGF.

Key words: Angiogenesis, Awar-awar leaf ethanol extract, Ficus septica Burm. f.

INTRODUCTION

Cancer is the leading cause of death after heart disease. The prevalence of cancer as a deadly disease increases every year (Taiwo et al., 2017). Cancer occurs as a result of a series of successive mutations in a gene so that these mutations change cell function. There are many causes of cancer, for example chemical compounds, viruses, bacteria, radiation rays, and other carcinogenic factors (Hassanpour dan Dehghani, 2017).

Angiogenesis is the growth of new blood vessels as a form of cell defense to obtain nutrients (Li et al., 2017). Angiogenesis plays a vital role in the growth and spread of cancers. Tumors stimulate healthy cells nearby to produce
molecules of angiogenesis signals. New blood vessels supply tumors grown with oxygen and nutrients, allowing cancer cells to invade nearby tissues, spread throughout the body, and form new colonies of cancer cells, called metastases (Katsi et al., 2014). Research on angiogenesis control shows the result that angiogenesis can play a role as a therapeutic agent (Adair and Montani, 2010). Inhibition of angiogenesis indicates the discontinuation of new blood vessel growth, resulting in hypoxic cells that eventually, tumor cells will be deprived of nutrients and die (Katsi et al., 2014).

Awar-awar (*Ficus septica* burm. F.) is a plant grown in Indonesia that has been reported to have potential as a chemopreventive agent (Sekti et al., 2010). Alkaloid content in awar-awar leaves shows potent cytotoxic activity in some cancer cells (Lansky et al., 2008). The awar-awar leaf ethanolic extract showed cytotoxic effects on MCF-7 cells having a IC₅₀ 6 μg/mL (Sekti et al, 2010), whereas in breast cancer with T47D line cells, it’s fractionated extracts containing alkaloids including indole alkaloid or its derivatives exhibited a cytotoxic effect (Nugroho et al., 2015). The awar-awar extract also exhibited combinational effects with doxorubicin on cell growth and apoptosis (Nugroho et al., 2013). An indole compound, tylophorine induced apoptosis in T47D breast cancer cells on administration alone and in combination with doxorubicin (Pratama et al., 2018).

**MATERIAL AND METHODS**

1. **Extraction of awar-awar leaves**

Awar-awar leaves were harvested from Sumber Arum, Moyudan, Sleman, Daerah Istimewa Yogyakarta. A total of 25 kg of freshly harvested leaves were sorted and washed and then were dried in an oven with a temperature of 60°C for 72 hours. The simplicia was powdered using a machine with a hole size of 0.75. 1.3 kg of awar-awar leaf powder was extracted with 13 L (1:10 v/v) of ethanol 70% v/v. Maserations were repeated twice to obtained the final ethanol-soluble extract. The extract was then thickened with a rotary evaporator with a temperature of 60°C at 60 rpm. After that, the macerate had been evaporated using a water bath until a thick ethanolic extract was obtained.

2. **Angiogenic assay**

Fertile eggs were collected from Java Farm Indo Asia in Yogyakarta, Indonesia. 0 day aged eggs were incubated for 48 hours at 37-39°C with constant humidity of 50%-60%, which was maintained by the addition of sterile distilled water to the incubator. Then the separation of eggshells was conducted. All of the eggs (excluding the shell) containing live embryos were preserved in a sterile glass bowl and incubated again. Tests were performed between the 6th to the 8th day of the incubation of the eggs. The experimental design consisted of two major groups, which were the control group and the treatment group. The control group consisted of 2 groups, namely group I: paper disc+30 ng/mL bFGF. This group was used as a negative control. Group II: standard tylophorine as a positive control. The group was treated with a paper disc+30 ng/mL bFGF+standard tylophorine concentration of 1×IC₅₀ (9.2 μM) (Saraswati et al., 2013). The treatment group consisted of 4 groups: group I: paper disc+30 ng/mL bFGF+13 μg/mL extract. Group II: paper disc+30 ng/mL bFGF+9.75 μg/mL extract. Group III: paper disc+30 ng/1 mL
bFGF+6.5 μg/mL extract. Group IV: paper disc+30 ng/mL bFGF+3.25 μg/mL extract. All treatments were tested on CAM chicken embryos maintained in an incubator at 37-39°C and constant humidity of 50%-60% for 48 hours. All treatments were tested in triplicate.

3. Observations and data collection

Observations were conducted after 48 hours of treatment. The number of new blood vessels or angiogenesis response in the egg culture was calculated. Macroscopic and microscopic observational methods evaluated the antiangiogenic effects. The macroscopic observation was performed by photo observation. The treated CAM was photographed from the dorsal and ventral directions, and the number of radial patterns of new blood vessels formed in each implant throughout treatment was calculated. The microscopic observations were performed on histologic slides. The implant area was fixed with Bouin solution for 3-4 hours. Then, it was cut using standard protocols with 6 μm thickness. The parts were stained with hematoxylin-eosin and Mallory’s fuchsin acid. The slides were examined under a microscope.

RESULT AND DISCUSSION

The result of the analysis with thin layer chromatography is shown in Figure 1. The result shows that there are alkaloid compounds in awar-awar leaves ethanolic extract. However, in the detection of 254 nm and 366 nm UV light, the spots are shown in the extract are not the same as the elution spots of the standard used (tylophorine). This can happen as not all alkaloid compounds are capable of fluorescence with 254 nm UV light. It is also possible that tylophorine content in awar-awar leaves ethanolic extract is tiny. Awar-awar leaves ethanolic extract in spraying with Dragendorff shows the result of red-brick spots which are as thin as the standard spots. Alkaloid compounds, if sprayed with Dragendorff reagent will become red-brick in color (Parbuntari dkk., 2018). Because of the possibility of awar-awar leaves ethanolic extract containing tylophorine in a small amount, another more sensitive method is needed to identify using the HPLC method. The result of LC-UV test (Figure 2) shows that the extract contains detected compounds at retention time of 3.123 minutes and on tylophorine at retention time of 3.371 minutes. Therefore, from these results, it indicates that awar-awar leaves ethanolic extract may not have alkaloids of tylophorine type as the peaks detected in the extract are not the same as the tylophorine in HPLC-UV identification.

Furthermore, it is also supported by thin-layer chromatography (TLC) data that the extract has different spot distance from tylophorine, both before and after spraying with Dragendorff reagent. The extract may have other alkaloids, which are still classified in the class of phenanthroindolizidine alkaloids. Many factors can cause differences in chemical content in natural materials. This is because plants are living organisms so that the geographical location or the place to grow plants, climate, method of cultivation, method and time of harvest, post-harvest treatment in the form of drying or storage can also affect the chemical content of herbal medicines (Hedi R. Dewoto, 2007).

Chemical compounds in awar-awar leaves ethanolic extract include phenanthroindolizidine, aminocapropanone, and pyrrolidine groups. The compounds that have active cytotoxic activity on NUGC and HONE-1 cells are tylophorine and ficuseptine, in which tylophorine and ficuseptine belong to the
phenanthroindolizidine alkaloid group (Damu et al., 2005). In addition, there are also Ficu septine A and 14α-hydroxyisocrobrine N-oxide compounds that have active cytotoxic activity against HeLa cells. There are also aminocapropanone groups (Ficu septamine A and Ficu septamine B) and pyrrolidine groups (Ficu septamine C) that do not have cytotoxic activity against HeLa cancer cells (Ueda et al., 2009). Therefore, from these results, it can be seen that the alkaloid compounds of phenanthroindolizidine group are a group of alkaloid which is responsible for cytotoxic activity awar-awar leaves.

The angiogenesis test with CAM is the most widely used in vivo test to study angiogenesis as it is relatively simple, inexpensive, and appropriate for large-scale screening (Staton et al., 2004). The macroscopic observation result is shown by the percentage of inhibition in Table 1, i.e. at the concentrations of 0.013 mg/mL of 62.30%, 0.00975 mg/mL of 51.37%, 0.0065 mg/mL of 28.42%, and 0.00325 mg/mL of 13.11%. The result is consistent with previous research on awar-awar (Ficus septica Burm F.) stems methanolic extract which shows that the methanolic extract of awar-awar stems is a potential antiangiogenesis agent because it can inhibit the growth of new blood vessels (angiogenic vessels) on duck embryo chorioallantoic membrane with a dose-dependent pattern. At the extract concentration of 6 mg/mL, the swelling occurred in the area that was given the extract, which resulted in bleeding and the Kupffer cells formed on the liver sinusoid. The Kupffer cell is an active component that shows that the extract correlates well enough with cytotoxic effects (Gamallo and Gallego, 2017). In this study, awar-awar leaves ethanolic extract also displays antiangiogenesis response on chicken embryo CAM. With the pattern of the higher dose used then, the more growth of new blood vessels inhibited, which is indicated by the higher dose, the pattern of radial blood vessel growth decreased. However, at specific doses that are too high can cause bleeding in chicken embryo CAM, which can eventually cause death in chicken embryos. In the study, the highest dose of CAM, which was 0.013 mg/mL gave inhibition power of 62.30%. The angiogenesis response in all test groups showed significantly different results for the bFGF control group.

The results of microscopic observation supported the data demonstrating the changes that occur in CAM blood vessels due to the treatment of test compounds. HE staining of paraffin identifies the ectoderm, represented by one or two cell epithelial layers; the capillary plexus is visualized as a small circular opening in the ectoderm which is often filled with erythrocytes; mesoderm which is filled with stromal cells, collagen fibres and blood vessels of different diameters, including terminal capillaries localized just below the ectoderm; and one layer of flat endodermic cells (Deryugina and Quigley, 2008). While coloring with MAF aims to visualize collagen, cytoplasm, and red blood cells (Goodfellow and Mikat, 2016), histology preparations can describe differences in the main blood vessels and new blood vessels from the process of angiogenesis. Vascular wall results from the angiogenesis process have thin walls with little collagen content compared to blood vessel walls resulting from the vasculogenesis process. A small red color indicates the HE coloring on the vessel wall resulting from angiogenesis and the MAF coloring appears thin blue.

From microscopic observation of Figure 4 and 5, there is a difference in the thickness of the walls of the central veins or blood vessels that existed before implanted with the test compounds (blood vessels resulted from the vasculogenesis process) of HE staining, which appears thicker than the walls of the new blood vessels (blood vessels resulted from the angiogenesis process).
The new blood vessels’ walls have a small collagen content from the preparation description with MAF staining, and it can be seen clearly in a visible thin blue color. The new angiogenic blood vessels were derived from the growth of blood vessels that had already existed. The angiogenic molecule induces angiogenic process, stimulates the degradation of the extracellular matrix, proliferation, migration and vascular formation of the vascular endothelial cells, and also regulates the permeability of the capillary walls, resulting in thinner angiogenic blood vessels, less collagen content, looser mesenchymal tissue, and decreased erythrocyte density (Adams and Alitalo, 2007).

Incubation of eggs at 36-40°C and 50% -60% relative humidity is the optimum condition to produce a good and long-lasting embryo (Lokman et al., 2012). Chicken eggs are opened on the 2nd day (48 hours). On the second day, the chicken embryo is formed. Incubation for 72 hours or longer has a significantly lower survival rate because, after 72 hours, the yolk pouch that has not been covered by CAM (chorioallantoic membrane) becomes thinner and tends to stick to the eggshell so that it can cause minor bleeding in certain areas and rupture of the yellow bag membrane (Dohle et al., 2009). Antiangiogenesis tests were carried out on chicken embryos aged six days. At the age of 6-10 days, the growth of blood vessels in the chorioallantoic membrane is at a high level, or at that age range, and blood vessels proliferate. On the 11th or 23rd day after incubation of the vascular system, the CAM will continue to decline and on the 18th day, the vascular system arrangement reaches the final stage (Ribatti, 2008).

Tylophorine of 9.2 µM/mL can inhibit the growth of new blood vessels or an antiangiogenesis agent. Tylophorine significantly inhibits VEGF-induced angiogenesis processes, including proliferation, migration, and endothelial cell formation (Saraswati et al., 2013). VEGF (Vascular endothelial growth factor) is one of the agents of proangiogenesis. Other proangiogenesis agents, bFGF (essential fibroblast growth factor). bFGF 30 ng/mL can effectively induce an increase in angiogenesis in CAM (Oktavia et al., 2017). bFGF is a polypeptide protein that is usually produced by the body. The bFGF protein has an essential role in blood vessel growth during chicken embryo development. bFGF in chicken allantoic fluid secretion. bFGF is a potential inductor compound in endothelial cell migration, cell proliferation, and new blood vessel formation (Terman and Stoletov, 2001).

New blood vessels observed are the vessels with radial growth patterns. Radial vessels in chicken CAM occur due to the induction of excess proangiogenesis agents. Cells respond by issuing a signal to initiate angiogenesis by secreting angiogenic growth factors immediately. Degradation of extracellular matrix and transfer of endothelial cells, the endothelial cell proliferation occurs, and new blood vessels emerge. Radial patterns are characterized by the growth of sprout-like vessels arranged from endothelial cells that grow towards angiogenic stimuli. Increased angiogenesis can increase the number of blood vessels to parts of the tissue that previously did not have blood vessels (Karthik et al., 2018).

The process of forming blood vessels is generally divided into two, namely vasculogenesis and angiogenesis. Vasculogenesis is the formation of blood vessels that develop from angioblast precursor cells, while angiogenesis grows from existing blood vessels. Angiogenic molecules encourage angiogenesis, but there are also specific inhibiting molecules to stop the angiogenesis process. Under normal circumstances, units with different functions will work continuously in balance to maintain a constant vascular system (Adams and Alitalo, 2007).
In healthy normal adults, endothelial cells change within years, but endothelial cells will proliferate rapidly (±5 days) when stimulation of angiogenesis occurs. When there are angiogenesis factors, this factor activates receptors on the cell surface that line the blood vessels. In Vitro, angiogenesis factors play a role in stimulating extracellular matrix degradation, proliferation, migration, and vessel cavities formation in vascular endothelial cells. Whereas in vivo regulates the permeability of capillary walls, which is vital in the initial process of angiogenesis. Blood vessels of angiogenesis, which are found in capillary tumor cells, have more excellent permeability than healthy cells, making them easier to leak (Adams and Alitalo, 2007). This is seen from histological preparations indicated by thin blood vessel walls.

In this research, it can be seen that the administration of awar-awar leaves ethanolic extract can inhibit the growth of new blood induced with bFGF. However, the mechanism of inhibitory action cannot yet be known for sure, because awar-awar leaves ethanolic extract itself contains various chemicals, including alkaloids. The phenanthro-indolizidin alkaloid is a compound responsible for cytotoxic activity in several cancer cells (Damu et al., 2005; Ueda et al., 2009). With these findings, it is possible that the alkaloid content of awar-awar leaves ethanolic extract can inhibit the growth of new blood vessels via the bFGF pathway.

CONCLUSION

Awar-awar leaves ethanolic extract has potential as an antiangiogenic agent. The macroscopic and microscopic observation indicates it with the less new blood vessels growth in implanted ethanolic extract of awar-awar leaves compared to the bFGF treated embryo.

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**TABLE AND FIGURE**

**Table 1. Angiogenic response and percent inhibition in CAM**

| Treatment   | Concentration (mg/mL) | Number of new blood vessels | Angiogenic inhibition (%) |
|-------------|-----------------------|-----------------------------|---------------------------|
| bFGF        | 0.000003              | 61.00 ± 5.57                | 0                         |
| Tylophorine | 0.00362               | 7.67 ± 0.58                 | 89.07 ± 0.95              |
Ekstrak of *Ficus septica* leaves

|          | 0.013  | 23,00 ± 2,65 | 62,30 ± 4,34 |
|----------|--------|--------------|--------------|
| 0.00975  |        | 29,67 ± 6,81 | 51,37 ± 11,16|
| 0.0065   |        | 43,67 ± 3,51 | 28,42 ± 5,76 |
| 0.00325  |        | 53,00 ± 3,46 | 13,11 ± 5,68 |

The values displayed as mean ± SD.

Figure 1. The images of TLC profile *Ficus septica* leaf extract. A: extract; B: tylophorine; 1: UV 254 nm; 2: UV 366 nm; 3: after spraying Dragendorff. The stationary phase: Silica gel 60 F254; Mobile phase: 2-propanol: glacial acetic acid: aquadest (5:1:1).
Figure 2. The images of HPLC profile *Ficus septica* leaf extract. A: extract; B: tylophorine; System: reversed-phase, Stationary phase: C18, mobile phase: methanol (100%), flowrate: 1mL/minute, detector: UV, wavelength: 275 nm.

Figure 3. The images of living CAMs implanted from ventral side at 8th days old. A: Paper disc + bFGF 30 ng/mL as a negative control; B: paper disc + bFGF 30 ng/mL + Tylophorine 9,2 µM/mL as positive control; C: paper disc + bFGF 30 ng/mL + extract 0,013 mg/ml; D: paper disc + bFGF 30 ng/mL + extract 0,00975 mg/ml; E: paper disc + bFGF 30 ng/mL + extract 0,0065 mg/ml; F: paper disc + bFGF 30 ng/mL + extract 0,00325 mg/ml; these images show the effect of awar-awar leaves extract on bFGF-induced angiogenesis in CAM. Star: implant paper disc; black arrows: main blood vessel, red arrows: new blood vessel.
Figure 4. Histological images of CAM and blood vessels with HE. 1: bFGF, 2: Thyrophorine, 3: Extract 0.013 mg/mL, 4: Extract 0.00975 mg/mL, 5: Extract 0.0065 mg/mL, 6: Extract 0.00325 mg/mL, black arrows: main blood vessel, red arrows: new blood vessel, magnification: 40x10.

Figure 5. Histological images of CAM and blood vessels with MAF. 1: bFGF, 2: Thyrophorine, 3: Extract 0.013 mg/mL, 4: Extract 0.00975 mg/mL, 5: Extract 0.0065 mg/mL, 6: Extract 0.00325 mg/mL, black arrows: main blood vessel, red arrows: new blood vessel, magnification: 40x10.