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Anti-angiogenic effect of *Artocarpus heterophyllus* seed methanolic extract in *ex ovo* chicken chorioallantoic membrane

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ABSTRACT

Objective: To examine the anti-angiogenic potential of *Artocarpus heterophyllus* (*A. heterophyllus*) seed extract in chicken chorioallantoic membrane (CAM).
Methods: This study used chicken CAM *ex ovo* culture to examine the potential anti-angiogenic activity of *A. heterophyllus* seed methanolic extract. Basic fibroblast growth factor was used to induce the ectopic formation of blood vessels on CAM treated with extract. Blood vessel number was assessed by macroscopic and microscopic observation, and compared and analyzed for all treatments and controls.
Results: Macroscopic observation revealed that a dose of 35 μg/mL of methanolic extract of *A. heterophyllus* seeds could inhibit basic fibroblast growth factor-induced angiogenesis by 61% in chicken CAM *ex ovo* culture. This concurred with microscopic observations on the histological structure of blood vessels, which indicated that extract treatment repressed the formation of new blood vessels.
Conclusions: This is the first study to report the anti-angiogenic effect of methanolic extract derived from *A. heterophyllus* seeds and its potential as a candidate for future anticancer therapy.

1. Introduction

Angiogenesis is the outgrowth of new blood vessels from pre-existing vessels. It commonly occurs during the normal physiological process of blood vessel formation and during cancer growth. There are many correlations between angiogenesis and cancer growth [1]. When cancer cells multiply and grow further away from the vessels that carry nutrient-rich blood, they may experience a lack of oxygen and nutrients [2]. In these conditions, cancer cells secrete tumour angiogenesis factors (TAFs) to trigger angiogenesis [3]. Basic fibroblast growth factor (bFGF) is one of the TAFs that play a role in angiogenesis [4]. As well as contributing to angiogenesis *in vivo*, bFGF can stimulate endothelial cell proliferation *in vitro* [5]. Overexpression of bFGF in tissue is indicative of the presence of cancer cells that are secreting TAFs to stimulate angiogenesis.

Compounds that can inhibit angiogenesis have great potential for cancer treatment [6]. Jackfruit seeds [*Artocarpus heterophyllus* Lamk. (*A. heterophyllus*)] contain secondary metabolites that display anticancer effects, especially anti-angiogenesis, and belong to the flavonoid group. Previous research reported that the methanolic fraction of jackfruit seeds contains flavonoids at concentration of up to (4.05 ± 0.01) mg/g [7]. These flavonoid compounds are present at levels higher than those of the components (acetone, ethyl acetate and water). The methanolic extract of jackfruit seeds has cytotoxic activity against the A549 cancerous cell line, but does not cause toxic effects in normal cells. The median inhibitory concentration (IC_{50}) values found for jackfruit seed methanolic extract against A549 cells in MTT and sulforhodamine B assays were 25.260 and 36.119 μg/mL, respectively [8]. Cytotoxicity toward cancer cells is probably caused by flavonoid compounds in jackfruit seed methanolic extract.

Experiments using *ex ovo* chicken chorioallantoic membrane (CAM) form the main part of this study. *Ex ovo* culture shows the short-term viability of the developing embryo in a Petri dish.

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This method is suitable for examining blood vessel development in CAM [9], by enhancing the accessibility and easy documentation of CAM to measure angiogenesis in embryo culture [10]. The effects of methanolic extract obtained from jackfruit seeds will be the main highlight of this paper, specifically to determine the optimum concentration for angiogenesis inhibition during chicken embryo growth.

2. Materials and methods

2.1. Extraction of jackfruit seeds

Jackfruit seeds used in this study were obtained from jackfruit trees planted in Bojanegara, Padamara District, Purbalingga, Central Java, Indonesia in December 2013. Ripe jackfruit seeds were collected and cut into small pieces before being dried in an oven at approximately 60 °C until they reached a constant sample weight. The dried samples were ground into a powder for methanolic extraction. The jackfruit seed powder extract was prepared using Soxhlet extraction for 50 cycles; the extract was then filtered and dried at room temperature.

2.2. Detection of active compounds

Active compounds in the jackfruit seed methanolic extract were detected by thin layer chromatography, which can identify active compounds such as flavonoids, saponins and tannins.

2.3. Jackfruit seed methanolic extract

Dry extracts of jackfruit seeds were dissolved in polyethylene glycol (PEG) 400 and phosphate buffered saline (PBS). These solvents have known biocompatibility properties including non-toxicity, hydrophilicity and high flexibility. Moreover, PEG 400 is a common solvent for tissue preparation [11].

2.4. Ex ovo culture preparation

Fertile eggs were collected from a hatchery in Yogyakarta, Indonesia. The eggs were incubated for 72 h at 37–39 °C with a constant humidity of 50%–60%, which was maintained by addition of sterile distilled water to the incubator.

After 72 h of incubation, the eggs were sterilized with 70% alcohol, marked for embryo location and then drilled to remove the egg shell. The whole egg (excluding the shell) was transferred to a sterile glass bowl and incubated in a sterile incubator. The CAMs were supplied with antibiotics and antimycotics at a dose of 10 000 units or using penicillin-streptomycin of 10% and 5%, with a total volume of 10 mL in sterile distilled water.

2.5. Angiogenesis assay

The assays were conducted between the 8th day and 10th day of egg incubation which is the period of maximum angiogenesis in CAM [12,13]. Experimental design was arranged for treatment group as follows. The control groups consisted of: 1) sterile paper disc implanted in CAM, 2) paper disc + PEG 400 + PBS, and 3) paper disc + 30 ng/1 μL bFGF. The treatment groups were paper disc + 30 ng/1 μL bFGF + methanolic extracts of jackfruit seeds at 17.50, 26.25, 35.00, 43.75 and 52.50 μg/mL. These extract concentrations represent 0.5×, 0.75×, 1×, 1.25× and 1.5× of IC_{50}, respectively. All treatments were tested on chicken embryo CAMs, which were maintained in an incubator at 37–39 °C for 72 h.

2.6. Preparation of bFGF

This study used 30 ng/1 μL bFGF concentration for each egg culture. The bFGF solution was made by using 1 μg/100 μL bFGF with 32.33 μL buffer Tris–HCl (10 mmol/L, pH 7.5), which resulted in 30 ng bFGF concentration in 1 μL bFGF. The levels of endogenous bFGF were significantly higher in CAM, ranging from 25 to 183 ng [14].

2.7. Observations and data collection

Observations were made at 72 h after implantation by carefully counting the number of new blood vessels or the angiogenesis response in the egg culture after treatment. The anti-angiogenic effect was evaluated by macroscopic and microscopic observation methods. Macroscopic observations were conducted by photo observation, where the treated CAMs were photographed and the number of new radial patterns of blood vessels formed in each implant during the treatment period was counted. Microscopic observations were conducted on histological slides, which were prepared by the paraffin method. The treated area of CAM was fixed with Bouin solution and sectioned using a standard protocol to a thickness of 6 μm, and the sections were stained with Mallory acid fuchsin. The slides were evaluated under a microscope for major blood vessels and new (angiogenic) blood vessels formed during the treatment.

2.8. Data analysis

Macroscopic and microscopic observation data were analyzed descriptively. Meanwhile, the data of angiogenesis inhibition in percentage were analyzed with non-parametric analysis of Kruskal–Wallis test by Duncan's multiple range test ($P < 0.05$) using SPSS software version 16.0 for Windows.

3. Results

Qualitative analysis by thin layer chromatography showed that the methanolic extract of jackfruit seeds contained several compounds including flavonoids, saponins and tannins. Results of macroscopic observation of the control group indicated that bFGF treatment induced aberrant formation of new blood vessels (Table 1, Figures 1 and 2), suggesting that bFGF applied to CAM

Table 1
Angiogenic response and percent inhibition in CAM with bFGF induction.

Treatment	Concentration of extract (μg/mL)	Number of new blood vessels	Angiogenic inhibition (%)
Paper disc + bFGF (30 ng)	–	36.00 ± 11.17^{c}	0^{a}
Paper disc + PEG 400 in PBS (v/v)	–	8.75 ± 4.57^{a}	0^{a}
Paper disc + bFGF + methanolic extract of *A. heterophyllus* seeds	17.50	26.50 ± 14.01^{c}	26.82 ± 23.17^{b}
	26.25	19.25 ± 6.50^{b}	46.68 ± 9.03^{c}
	35.00	14.00 ± 7.62^{b}	61.14 ± 15.73^{d}
	43.75	15.00 ± 9.93^{b}	62.40 ± 17.78^{d}
	52.50	10.75 ± 1.50^{a}	68.35 ± 8.14^{d}

The values displayed as mean ± SD. Values in the same column with a different superscript differ significantly ($P < 0.05$).

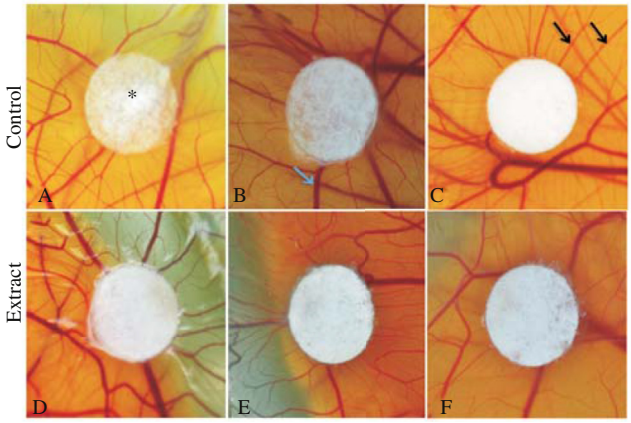

Figure 1. Images of live CAMs implanted with bFGF and different concentrations of jackfruit seed extract.
A: Paper disc only implant as a negative control; B: paper disc + PEG 400 + PBS as another negative control; C: bFGF implant; D: 17.50 μg/mL extract; E: 35.00 μg/mL extract; F: 52.50 μg/mL extract. These images show the effect of jackfruit seed extract on bFGF-induced angiogenesis in CAM. Blue arrow: main blood vessel; black arrows: new blood vessels; asterisk: paper disc.

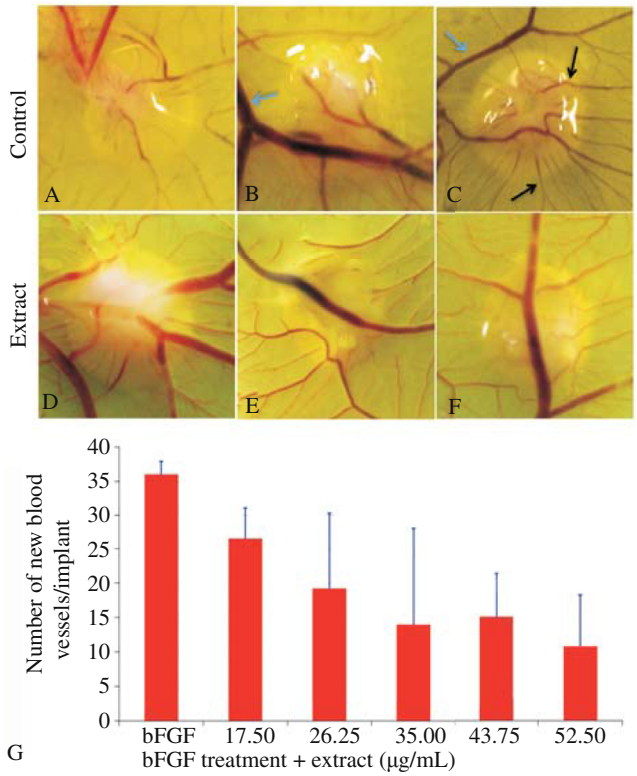

Figure 2. CAMs implanted with bFGF and different concentrations of jackfruit seed extract, and fixed with Bouin solution.
A: Paper disc only implant as a negative control; B: paper disc + PEG 400 + PBS; C: bFGF implant; D: 17.50 μg/mL extract; E: 35.00 μg/mL extract; F: 52.50 μg/mL extract, respectively; G: graph of number of blood vessels in positive control and different treatment CAMs, showing the inhibition of angiogenesis by extract at specific doses. Blue arrows: main blood vessel; black arrows: new blood vessel.

mimics the role of bFGF released by cancer tissue *in vivo*, as one of the control agents in angiogenesis. Controls with paper disc only and paper disc + PEG 400 + PBS revealed a smaller number of newly formed blood vessels (7.75 ± 1.89 and 8.75 ± 4.57, respectively), compared to 36.00 ± 11.17 new blood vessels in paper disc + bFGF (Table 1).

Macroscopic observation and quantification of new blood vessels revealed that all treatments inhibited the bFGF-induced formation of new blood vessels in CAM. Moreover, in terms of percent inhibition of angiogenesis, all treatments resulted in significant inhibition ($P < 0.05$). CAM implanted with bFGF together with 17.50 or 26.25 μg/mL extract, showed a moderate degree of angiogenesis inhibition of 26.82% ± 23.17% or 46.68% ± 9.03%, respectively, while the higher concentrations of 35.00, 43.75 and 52.50 μg/mL inhibited angiogenesis more strongly, by 61.14% ± 15.73%, 62.40% ± 17.78% and 68.35% ± 8.14%, respectively (Table 1).

Histological examination showed two different blood vessel types in CAM: thick-walled blood vessels with dense erythrocytes and thin-walled vessels with fewer erythrocytes. Blood vessels in the negative control (Figure 3A,B) had a large diameter, a thick wall and dense erythrocytes, whereas bFGF control vessels (Figure 3C) had many small, thin-walled blood vessels with a small number of erythrocytes. On the other hand, the treatment group (Figure 3D,E,F) had a smaller number of small and thin-walled blood vessels compared to the bFGF control, in a dosage-dependent manner.

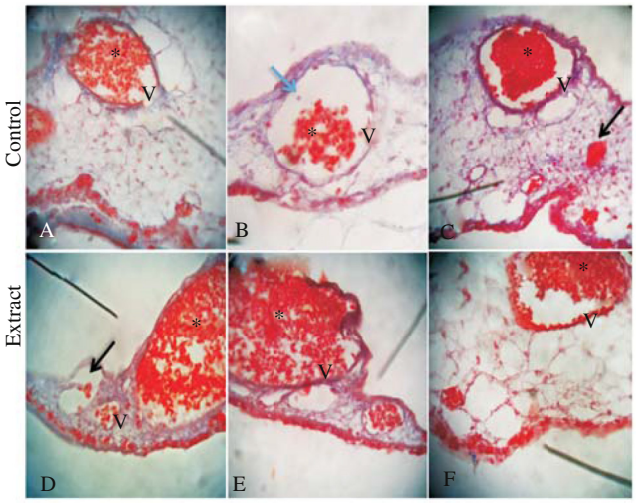

Figure 3. Histological images of CAM and blood vessels.
A: Paper disc only implant as a negative control; B: Paper disc + PEG 400 + PBS; C: bFGF implant; D: 17.50 μg/mL extract; E: 35.00 μg/mL extract; F: 52.50 μg/mL extract, respectively. Blue arrow: main blood vessel; black arrows: new blood vessel; asterisk: erythrocyte; V: vascular wall.

4. Discussion

Methanolic extracts of jackfruit seed (*A. heterophyllus*) contain flavonoids, tannins and saponins (terpenoids) [8,15]. Flavonoids, saponins and tannins are compounds which have potential as cell growth inhibition, anticancer and anti-angiogenesis agents [16–20].

CAM has natural endogenous bFGF derived from chicken allantoic fluid secretion. This protein plays an important role in the growth of blood vessels during development of the chicken embryo [19]. Observation of the bFGF-untreated control group showed no significant increase of new blood vessels, in contrast to the bFGF-treated control group. Therefore, the significant increase of new blood vessels in the bFGF control group suggests that the added bFGF was the only factor which could have stimulated the ectopic angiogenesis in CAM. This result also

indicates that a dose of 30 ng of bFGF was effective in inducing an increase of angiogenesis in CAM. It has been reported in previous research that there was no ectopic angiogenesis in CAM in the absence of bFGF administration [4].

Extract addition in the treated group showed that concentrations of 17.50 and 26.25 μg/mL were not highly effective in inhibiting angiogenesis, whereas concentrations of 35.00, 43.75 and 52.50 μg/mL inhibited angiogenesis by more than 60%, which is very effective. However, application of extract at concentrations higher than 35.00 μg/mL showed no significant difference in inhibition. This indicates that the effective dose of extract for angiogenesis inhibition is 35.00 μg/mL. The results suggest that higher concentrations of methanolic extract of jackfruit seeds implanted in CAM generally led to higher levels of angiogenesis inhibition. However, based on our preliminary studies, implants with extract concentrations higher than 52.50 μg/mL appeared to be toxic to the embryo.

Histological examination of CAM in the bFGF control showed a high density of small, thin-walled blood vessels with less dense erythrocytes in contrast to fewer and thick-walled blood vessels with dense erythrocytes in the extract-treated group. These findings indicate that in the bFGF control, several new blood vessels formed, induced by bFGF treatment. On the other hand, angiogenesis induction by bFGF was inhibited by the extract in the treatment group.

From the active compound detection test, the nature of the compound responsible for anti-angiogenic activity in the tested extracts and a possible mechanism for this activity were not identified. The combination of flavonoids, saponins and tannins in the extracts may have inhibited the activity of growth factor bFGF. Methanolic extracts of jackfruit seeds were found in a previous study to be capable of inducing apoptosis in cell culture, where the authors associated with the mechanism that inhibits endothelial cell migration and proliferation of new blood vessels [8]. Flavonoid compounds in jackfruit seeds could probably suppress growth factors without harming endothelial cells that had been established [17,21]. Another compound with saponins and tannins was reported to possess anti-angiogenic properties. Saponins of *Panax ginseng* significantly inhibited proliferation, adhesion and migration of endothelial cells and capillary tube formation induced by TAFs, especially bFGF [22,23]. The saponins of *Momordica cymbalaria* reduced vascular branching in CAM [24], and tannins from *Phyllanthus urinaria* can suppress angiogenesis by up to 74% in CAM through a mechanism involving matrix metalloproteinase [18,25]. Nevertheless, the present findings indicate that further in-depth study is required to reveal specific details regarding the identity and mode of action of secondary metabolites that are involved in angiogenesis inhibition.

Conflict of interest statement

We declare that we have no conflict of interest.

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