

Antiangiogenic Activity of Pineapple (*Ananas comosus*) Stem Extract on Chicken Embryo's Chorioallantois Membrane (CAM)

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Abstract. Inhibition of angiogenesis is able to suppress cancer growth by starving the cancer cells. It has been reported that the growth of human tongue squamous cell carcinoma can be inhibited by administering pineapple's (*Ananas comosus*) extract. However, antiangiogenic activity of this extract has not been studied yet. This study aimed to investigate antiangiogenic activity of pineapple's stem extract on chorioallantoic membrane (CAM) of chicken embryo. Pineapple stems were extracted by ultrasonic-assisted method using ethanol 96%. The chemical compositions were determined by thin layer chromatography (TLC) and the protein concentration was analysed by the biuret method. In-ovo antiangiogenics assay was performed on CAM induced by basic fibroblast growth factor (bFGF). The extract at concentrations of 0.6%, 0.9% and 1.2% were administered on days 9-14 of egg incubation. We counted the number of CAM vasculatures using a stereomicroscope and examined the embryonic blood smears-stained May-Grunwald to investigate the extract-induced inflammation. Pineapple extract contained saponin by TLC and 1.93 mg/ml protein by the biuret test. The vasculatures were significantly reduced by all concentrations of the extract. At a concentration of 1.2%, the extract did not induce notable inflammation in chicken embryos. In conclusion, pineapple stems extract shows antiangiogenics activity on CAM.

1 Introduction

Angiogenesis is the formation of new blood vessels from existing vascular structures. It plays a physiological role in organogenesis, wound healing, and developing collateral circulation to improve organ perfusion. Nevertheless, abnormally accelerated angiogenesis is associated with pathological processes, including cancer. Angiogenesis is a crucial step for the development and growth of solid tumours beyond 2 – 3 mm³. It aids in supplying nutrients to promote tumour growth and sustain the dissemination of tumour cells [1, 2]. Tumour angiogenesis is mediated by the interaction between angiogenesis factors secreted by the

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tumour and endothelial surface receptors. Angiogenesis contributes to cancer growth and metastasis, therefore angiogenesis inhibitor or antiangiogenesis agent is a potential option for cancer therapy. Cancer therapies that directly kill cancer cells are considered less effective because the carcinogenic compounds of the cells are still able to spread freely to other tissues through blood vessels. On the other side, antiangiogenesis therapy completely blocks the supply of nutrients and oxygen to cancer so the cancer cell growth is inhibited. Therefore, cancer therapy with antiangiogenesis is more effective in curing the disease than directly killing cancer cells [3].

Dietary components have been shown to act as chemopreventive agents and thus have the potential to be an element of cancer treatment [4]. Some natural plant-derived compounds are able to inhibit the proliferation and growth of the cancer cells and prevent the formation of new blood vessels in the cancer [5]. Pineapple (*Ananas comosus*) is a fruit crop that is available throughout the year and has been widely grown in Indonesia. Pineapple stem is a waste product that contributes 20% of the total waste generated by the pineapple processing industry [6]. Ethanol extract of pineapple stem has been shown to have cytotoxic effects on human tongue squamous cell carcinoma. The IC₅₀ of the ethanol extract of pineapple stem was 6,324.49 ppm. Pineapple extract has been known as the main source of bromelain enzyme [7]. Previous studies show that bromelain can inhibit nuclear factor kappa B (NF- κ B) and cyclooxygenase-2 (COX-2) [8]. COX-2 has a major role in oncogenesis. Elevated synthesis of COX-2 and PGE2 inhibit Bax-triggered apoptosis in cancer. Additionally, increased COX-2 expression stimulates tumour angiogenesis by promoting pro-angiogenic factors and directly influencing endothelial cells [9]. Therefore, pineapple extract rich in bromelain has the potential to be studied as an antiangiogenic agent.

In this study, we investigated the antiangiogenic effects of pineapple stem extract. Antiangiogenesis acts on several proteins that have been identified as angiogenesis factors to inhibit blood vessel formation. The balance between activators and inhibitors of angiogenesis is important to maintain vascular homeostasis [2]. The blood vessel network leading to cancer cells was modelled using the blood vessel network found in the chorioallantoic membrane (CAM) of chicken embryos. The CAM is a thin, vascular-rich structure that lies beneath the eggshell. This structure has several advantages for research to study and manipulate blood vessels due to its easy accessibility, visibility, and rapid development [3]. Therefore, this study aims to assess the antiangiogenesis effect of pineapple stem extract on the CAM of chicken embryos in-ovo to observe the reduction in the number of blood vessels formed. The null hypothesis (H0) of this study is that the pineapple extract will not have any effect on the number of blood vessels, either increasing or decreasing them compared to the bFGF control. Meanwhile, the alternative hypothesis (H1) of this study is that the pineapple extract will normalize the number of blood vessels or has an antiangiogenic effect on the CAM compared to the bFGF control.

2 Methods

This research was conducted in April – August 2019 and took place at the Laboratory of Phytochemical – Pharmacognosy, Faculty of Pharmacy, Universitas Gadjah Mada for the extraction, the Laboratory of Analytical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada for quantitation of protein using biuret test, and the Laboratory of Animal Structure and Development of the Faculty of Biology, Universitas Gadjah Mada for antiangiogenic test.

2.1 Materials

Required materials were oven, dry blender, erlenmeyer, funnel, ultrasonic bath, cup, refrigerator, test tube, 25 mL flask, micropipette, analytical balance, microtube, UV-vis spectrophotometer, UV cabinet, incubator, pencil, autoclave, laminar air flow (LAF) hood, micro tweezers, micro scissors, stereo microscope, DSLR camera, flashlight, glass dish, petridish, mini drill, objective glass, pipette, Leica DM750 microscope connected to Leica ICC50 E camera, pineapple stem, 96% of ethanol, bovine serum albumin (BSA), biuret reagent, 0.1 M phosphate buffer pH 7.5, distilled water, rutin, saponin, quinin, gallic acid, ethyl acetate, formic acid, glacial acetic acid, chloroform, methanol, toluene, diethylamine, cytoborate, liberman bourchat, dragendorff, FeCl_3 , silica gel plate 60 F254, super embryonated Java chicken eggs, aluminium foil, bFGF, paperdisc, DMSO solvent, 70% of ethanol, paraffin, 0.9% of NaCl solution, and neutral buffered formalin (NBF), methanol, and May-Grunwald dye.

2.2 Ultrasonic Assisted Extraction of The Pineapple

Pineapple stems were dried in an oven at 50°C. The dried sample was crushed into powder and dissolved in 96% of ethanol with a ratio (1:10) in an erlenmeyer. The mixture was exposed to ultrasonic waves with 1.5 m of amplitude, 50 kHz of frequency, and 360 W of power for 40 minutes at 25°C. The remaining ethanol was evaporated at room temperature until a concentrated extract was obtained. The solid extract was made in several variations of concentrations based on the number of IC_{50} and multiplied by 1, 1.5, and 2, so the variations of concentration were 0.6%, 0.9%, and 1.2% [7].

2.3 Thin Layer Chromatography (TLC)

A stock solution was made by dissolving 70 mg of pineapple extract in 0.5 ml of 70% ethanol. The solution was homogenized using a vortex mixer. The stock solution was applied using a capillary tube onto the silica gel along with the standard, and then was dried. Silica gel was eluted in a chamber containing the mobile phase that has been saturated with filter paper first. After the mobile phase had reached 1 cm below the top end, the silica gel plate was removed from the chamber and dried. The silica gel was sprayed by the detectors then identified according to the character of the compound.

2.4 Quantitative Determination of Protein using the Biuret Method

A standard solution was prepared by dissolving 25 μg of bovine serum albumin (BSA) in 25 ml distilled water. . The water was added gradually to avoid foam. A stock solution was prepared by dissolving 0.25 g of pineapple extract in 0.5 ml of phosphate buffer. BSA standard solution, blanks, and pineapple extract stock solution was mixed with biuret reagent and 0.1 M phosphate buffer pH 7.5. All solutions were incubated for 10 min, then the absorbance was measured at 550 nm using UV-vis spectrophotometry.

2.5 In-ovo Treatment on Chicken Eggs

All tools used in the second step were sterilised using autoclave and the laminar air flow (LAF) hood was cleaned using 70% of ethanol then sterilised using UV light for 2 hours. The in-ovo procedure has received ethical approval from the Ethics Committees of the Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada, Indonesia (KE/FK/0487/EC/2019). The embryonic eggs were incubated for 9 days at 39°C and 50% –

65% of humidity with horizontal position and without rotation, ensuring that the CAMs were at the top of the eggs. The existence of embryos and their position were checked using a flashlight. The eggshell was perforated at the air sac and CAM region horizontally. The air cell was suctioned until it moved to the upper side of the CAM. The eggshell around the horizontal axis hole was thinned using a mini drill until the membrane was visible. Inside the LAF hood, egg membrane was cut out to form a window. The paperdisc containing the treatment was carefully placed on CAM using sterile tweezers, specifically in the area between the two blood vessel branches. The paperdisc should not be placed on embryos. The eggs were incubated again for 5 days.

2.6 Harvesting CAM After In-ovo Treatment

Albumin, egg yolk, and embryo were removed without changing the position of the paperdisc. CAM was stretched on a watch glass that had been soaked with 0.9% of NaCl solution. Filter paper was attached to the edge of the CAM to maintain its position. The CAM was then photographed using a DSLR camera. During the documentation process, CAM must be kept wet by 0.9% of NaCl solution. The number of blood vessel on paperdisc was manually counted.

2.7 Blood Smear Preparations

Embryonic blood samples were collected from blood vessels that were unaffected by the paperdisc implant. The blood was smeared along the objective glass and allowed to air dry. The area of the blood smear was covered by methanol for 5 minutes. Subsequently, the remaining methanol was dried. The area of the blood smear was covered by May-Grunwald staining, which had been dissolved with distilled water (1:1), for 15 minutes. The remaining stain then was rinsed by distilled water. The blood cells were photographed using a Leica DM750 microscope connected to a Leica ICC50 E camera.

2.8 Data Analysis

Data analysis was conducted using Statistical Package for Social Sciences (SPSS) v.26.0 software. The numeric data of vascular numbers were tested normal and homogeneous, so the data were analysed by one-way ANOVA and Tukey post hoc test. The nominal data of leukocyte type percentages were analyzed using the Chi-Square test. The confidence value was 95% with p-value <0.05. Data are presented as mean $\pm$ standard deviation.

3 Result and Discussion

3.1 Identification of Bioactive Compound in Pineapple Extract

Based on Figure 1, pineapple extract contains saponins. The retention factor (Rf) of saponins is 0.32, so it is considered ideal within the range of 0.2 to 0.8. Rf is affected by the polarity of the mobile phase. Rf below 0.2 indicates that the spots cannot elute well because the mobile phase used is not able to separate the compounds, while Rf above 0.8 means that the compound has reached its elution limit that the spots may still be separated further [10].

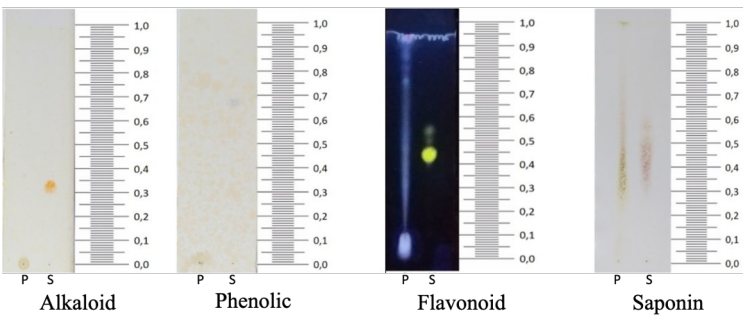


Fig. 1. Thin layer chromatogram. P: pineapple extract, S: standard

The pineapple stem extract contains various cysteine proteases (iso)forms of the papain family and other non-proteolytic compounds. The most widely identified cysteine protease is basic stem bromelain [11]. Bromelain concentration was determined by the biuret test. A standard curve was generated by plotting absorbance values at 550 nm with the concentrations of BSA as the standard. This produced a linear equation of $y = 0.1745x + 0.0901$, where y represents the absorbance value at 550 nm and x represents the protein concentration in mg/ml. (Figure 2). It was found that the total protein concentration in pineapple extract was 1.93 mg/ml as presented in Table 1.

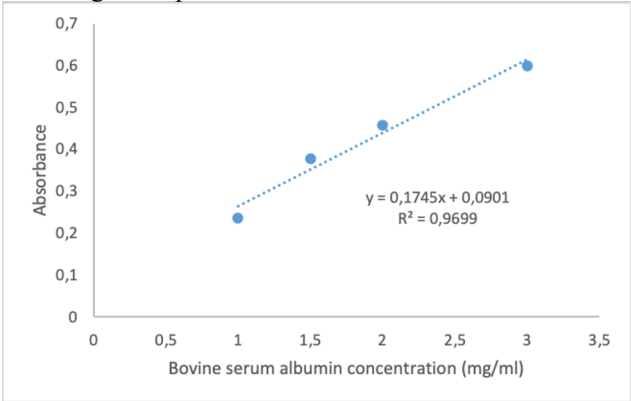


Fig. 2. BSA calibration curve

Table 1. Results of sample spectrophotometry and protein levels.

	Absorbance	Concentration (mg/ml)
Sample 1	0.458	2.11
Sample 2	0.397	1.76
Average		1.93 ± 0.247

3.2 Antiangiogenic Activity

In Figure 3, bFGF induced the angiogenesis process in CAM, which was characterised by a significantly higher number of blood vessels in the bFGF control compared to the paperdisc control without bFGF. Based on Figure 4, DMSO DMSO+bFGF and bFGF groups had no statistical difference. This indicates that the use of DMSO as a solvent had no significant

effect on the angiogenesis process. Both DMSO+bFGF and bFGF groups were highly vascularized and showed numerous thin new blood vessel growth towards the implants.

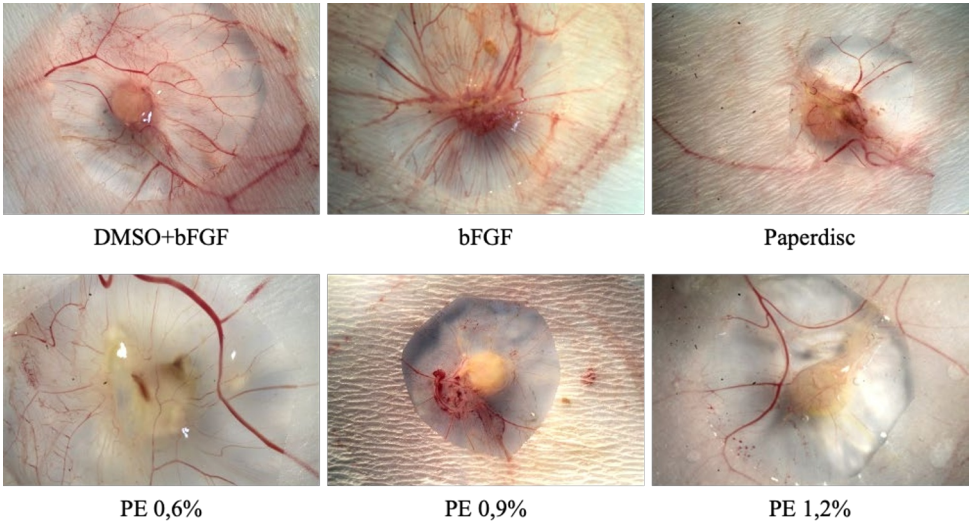


Fig. 3. Representative images of CAM upon exposure to various treatment groups. DMSO+bFGF: bFGF-induced CAM in DMSO solvent, bFGF: bFGF-induced CAM, paperdisc: CAM without treatment, PE 0,6%: pineapple extract 0.6%, PE 0,9%: pineapple extract 0.9%, PE 1,2%: pineapple extract 1.2%

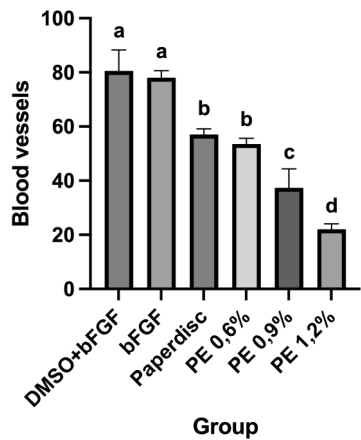


Fig. 4. Vascular count of CAM. PE 0,6%: pineapple extract 0.6%, PE 0,9%: pineapple extract 0.9%, PE 1,2%: pineapple extract 1.2%. Different letters indicate statistically significant differences (P<0.05)

The vasculatures were significantly reduced by all concentrations of the pineapple extract in a dose-dependent manner based on Figure 3. Antiangiogenic activity of pineapple extract was indicated by fewer blood vessels and no new blood vessels growth towards the implants compared to the bFGF-DMSO group in Figure 4. The percentage of vascular reduction in chorioallantoic membrane after exposure by pineapple extract at concentrations of 0.6%, 0.9%, and 1.2%, respectively were 33.5%; 49.1%; and 72.7%. The highest antiangiogenic activity was shown by pineapple extract of 1.2%.

Based on previous tests in this study, pineapple extract was proven to contain saponin and protein including bromelain. An earlier study showed that the compound saponin from

green tea demonstrated antiangiogenic effect in human umbilical vein endothelial cells (HUVEC) due to the suppression of the phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), vascular endothelial growth factor receptor-2 (VEGFR-2), and NF- κ B [12]. Another study showed that bromelain inhibits COX-2, inactivates NF- κ B, and reduces extracellular signal regulated protein kinase (ERK1/2), p38 mitogen-activated protein kinase (MAPK) and Akt in tumour-initiating effects in 2-stage mouse skin tumorigenesis model [13]. The inhibition of these mediators contributes to antiangiogenesis activity in cancer [14]. These findings suggest the synergistic antiangiogenic effect of saponin and bromelain through mutually inhibiting Akt and indirectly through inhibition of pro-inflammation NF- κ B.

3.3 Leukocyte Identification in Chicken Embryo Blood

Embryonic blood smear slides were stained by May-Grunwald for identification of leukocyte types (Figure 5). The reaction of chicken embryo immunity can be observed on day 14. That is because thymus cells have appeared since day 11 and cell-mediated immunity appears on day 13 to day 14. On day 12, mononuclear phagocytes were found in egg yolk, lymph, intestines, thymus and liver [15].

Based on Figure 6, chicken embryos in the pineapple extract 1.2% group did not exhibit a higher percentage of heterophils, lymphocytes, and monocytes compared to the paperdisc groups. Avian heterophils are equivalent to neutrophils in mammals, both have many similar functions. Heterophils and monocytes both are major mediators of innate immune response through phagocytosis. Lymphocytes play an important role in cellular and humoral adaptive immune response [16]. The increase of percentage in eosinophils and basophils were observed in the pineapple extract 1.2% groups compared to the paperdisc groups. However, the higher percentage of eosinophil was not an indication of inflammation. Avian eosinophil was not functional due to lack of IL-5 expression and IgE production. Avian basophils appear to have similar functions to mammalian basophils [16]. Yet, the cause of the elevation of basophils in chicken embryos in the pineapple extract 1.2% groups compared to paperdisc groups remains unclear. Thus, at a concentration of 1.2%, the extract did not induce notable inflammation in chicken embryos.

The embryo age is an important confounding variable in leukocyte type count. The immune system of chicken embryos appears to be completely functional between 12 to 18 days [16]. A recent study showed that the white blood cell count and inflammation biomarkers did not change after induction of figastrin in chicken embryos at 10 and 12 days of age [17]. This is probably due to the immature immune system of chicken embryos.

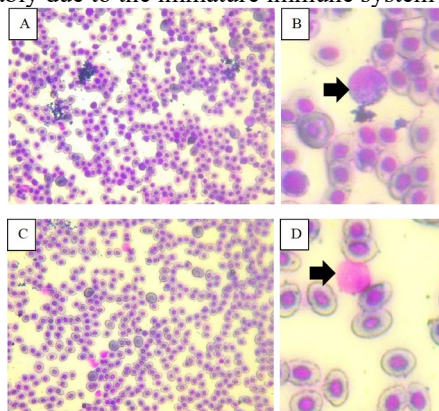


Fig. 5. Blood smear of chicken embryos with May-Grunwald staining A: blood smear of paperdisc control, B: heterophil on blood smear of paperdisc control, C: blood smear of pineapple extract 1.2%, D: eosinophil on blood smear of pineapple extract 1.2%

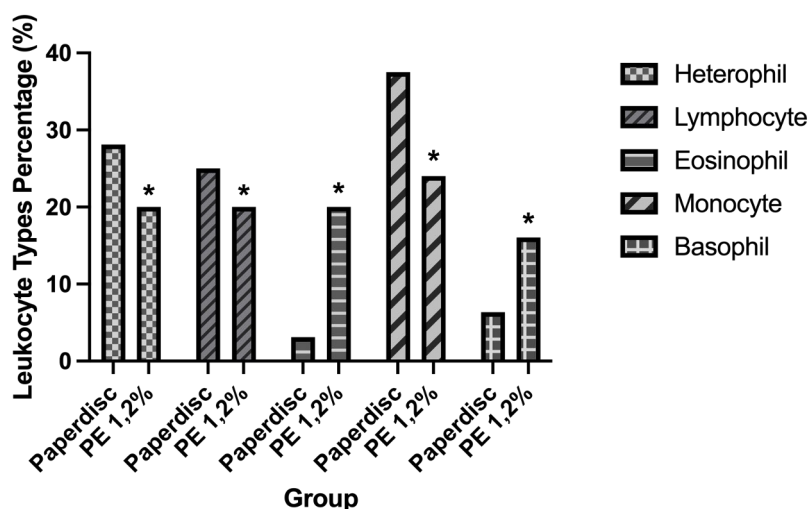


Fig. 6. Percentage of leukocyte types in blood of chicken embryos treated by paperdisc control and pineapple extract 1.2% (PE 1,2%). * $p < 0.05$ vs paperdisc

4 Conclusion

According to the results, it was found that pineapple extract contained saponin by TLC and 1.93 mg/ml protein by the biuret test. The vasculatures were significantly reduced by all concentrations of the extract. The percentage of vascular reduction in chorioallantoic membrane after exposure by pineapple extract at concentrations of 0.6%, 0.9%, and 1.2%, respectively were 33.5%; 49.1%; and 72.7%. At a concentration of 1.2%, the extract did not induce inflammation in chicken embryos. Before being applied in cancer therapy, several tests need to be conducted first, such as a toxicity test to determine the safe dose limit and effectiveness test to determine the effectiveness dose and side effects of pineapple extract.

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