



Anti-Angiogenesis activity of acetone extract of *Ananus comosus* by using Chorioallantoic membrane (CAM) assay Technique

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ABSTRACT

Background: Nowadays Herbal compounds uses in the treatment of several disease were significantly increased. **Aim and objective:** This study was done to evaluate the antiangiogenic activity of acetonic extract of *Ananus comosus* using chick chorioallantoic membrane (CAM) model. **Materials and methods:** The Antiangiogenic activity of *Ananus comosus* 1,2,3,4 and 5 µg/egg in chick embryos was assessed using chick CAM assay. Acetone was used to extract the herbal compound. **Results:** The outcome of the study confirmed that *Ananus comosus* contains active constituent like (Flavonoids, Bromelain, Beta-carotene, Vitamin-A and Antioxidants), which possess angiogenic activity and these used as pain and swelling, especially of the nose and sinuses, gums, and other body parts after surgery or injury. It was exhibited angiogenic activity by triggered neovascularization in concentration dependent manner. **Conclusion:** We concluded that the fruit possess significant antiangiogenic activity and new blood vessels were inhibited in the chick embryos.

Keywords: *Ananus comosus*, Bromelain, Antiangiogenesis, CAM assay, Chick embryos, Neovascularization.

INTRODUCTION

Cancer is a disease in which abnormal cells divide uncontrollably and destroy body tissue. Cancer has the ability to spread to adjacent or distant organs, which makes it life threatening. Tumour cells can proliferate at another site: metastasis. For the metastatic spread of cancer tissue, growth of the vascular network is important. The processes whereby new blood are called angiogenesis and lymphangiogenesis, respectively. Both have an essential role in the formation of new vascular network to supply nutrients oxygen and immune cells, and also to remove waste products [1-5]. The term angiogenesis defines the angio means 'Blood vessels' and genesis means 'Creation'. Angiogenesis is the formation of new blood vessels, an essential process that facilitates tissue growth and wound healing in living things. It is important in some physiological conditions such as ovulation, embryo development, wound healing and other disease conditions such as diabetic retinopathy, arthritis and tumours.

However diseases like cancer can take advantage of angiogenesis and use it to grow a spread. Tumour growth and metastasis depend on angiogenesis and lymphangiogenesis triggered by chemical signals from tumour cells in a phase of rapid growth [2, 6-9]. Recruitment of new blood vessels plays an important role in tumour survival and growth. In 1971, a concept of antitumour strategy by controlling angiogenesis was hypothesized by Folk man, as tumour growth is angiogenesis dependent and every aspect of tumour growth requires increment in vascular growth [3, 4, 10-16]. Endothelial cells in tumour bed tend to be more susceptible to cytogenic agents due to their high proliferation rate. In addition, endothelial cells on the contrary to cancerous cells are genetically stable as they do not undergo mutations and hence more sensitive to apoptotic effects of the cytotoxic agents [5, 17-21]. Highly vascularized chorioallantoic membrane of the chicken embryo is used as in vivo model and widely used for the determination of anti-angiogenesis activity. Due to the increasing interest in anti-angiogenic therapy for cancer several agents are investigated which act as angiogenesis inhibitors [6, 22-29].

Plants with anti-angiogenesis properties are therefore of considerable importance for diseases such as cancer, macular degeneration, diabetic retinopathy, and others [30-39]. Plants have been used in the treatment of cancer since ancient period. Natural products are related drugs are used to treat 87% of all human diseases including cancer. About 25% of prescribed

drugs in the world are obtained from various plants. Over 3000 species of different medicinal plants have been reported to have anti-cancer properties [40-45]. Ayurvedic system of medicine reveals that there are several herbs with anticancer and anti-inflammatory activity [12, 48-51]. Phytochemicals like polyphenols, flavonoid, catechins [52, 53]. Currently over 60% of used anti-cancer agents are derived in one or the other way from natural sources, including plants, marine organisms and microorganisms. There are worldwide efforts to discover new anticancer agents from plants [54-59].

Ananas comosus (Family Bromeliaceae) is known as pineapple in English, a fruit with 30-40 stiff succulent leaves closely spaced in a rosette on a thick fleshy stem. Bromelain is a group of enzymes found in the fruit and stem of the pineapple plant [16, 60-62]. Bromelain is a mixture of enzymes found in pineapples (*Ananas comosus*) that digest protein. Pineapple has been used for centuries in Central and South America to treat indigestion and reduce inflammation. Bromelain, which is derived from the stem and juice of the pineapple, was first isolated from the pineapple plant in the late 1800s. The German commission E approved bromelain to treat swelling and inflammation after surgery, particularly sinus surgery. Bromelain can be used to treat a number of conditions, but it is particularly effective in reducing inflammation from infections and injuries, pain after surgery, Tendinitis and physical injuries. Bromelain also contains chemicals that seem to interfere with tumour cells and slow blood clotting [17, 63, 64].

MATERIALS AND METHODS

Collection of plant Materials and Authentication:

The fruit selected for the studies is *Ananas Comosus* which is available plenty as a eatable fruit everywhere. The fleshy part of the fruit is taken for studies and whole fruit is shown up for the authentication. All the plant materials were collected from Kolli Hills, Namakkal district of Tamilnadu. The collected plant materials were preserved for the preparation of specimens and the same was authenticated by Kandaswami Kandar college (Department of Botany), P.Velur and the authenticated certificates were obtained. After that, the flesh part of the fruit were made into small pieces for easy drying in the shade. To avoid the dust contamination, the materials were covered with thin transparent cloth.

Materials

- Acetone bought from Molychem.

Preparation of Extract

The extract was prepared, the fleshy part of the fruit was dried and they were pulverized into coarse powder for the easy extraction and also help the penetration of solvents to dissolve the active constituents present inside the cells. Extraction technique chosen was a Maceration technique, in which the dried constituents were added with 500ml of Acetone. The solvent was kept 72 hours with continuous stirring for every 4 hours. Then the solvent is evaporated using Heating mantle. Afterwards the evaporated sample is diluted with Distilled water and used for further studies¹⁸.

SCREENING METHOD

In vivo anti-angiogenic assay: (Chick chorio allantoic membrane (CAM) model): The chick chorio allantoic membrane (CAM) model is used to compare the in-vivo antiangiogenic activity of the sample with processed procedure¹⁹. Fertile Country Chicken eggs were obtained from local hatchery.

Inclusion criteria:

- Three days old fertilized, medium sized healthy Country
- chick embryos
- Without crack / Crack free embryos
- Experiment should be simple to perform and uniformly
- reproducible
- Technique should be able to predict the potential
- properties of standard drug
- It should consume minimal quantities of drugs

Exclusion criteria:

- More than three days old fertilized chicken embryos
- Large size embryos
- Diseased chick embryos
- Embryos with crack
- Double yolk embryos

In the present investigation, a total 60 eggs were taken and divided into 6 groups of 10 eggs each.

- Group-1(control)
- Group-2-6(various concentration of the AE)

Briefly, fertilized chicken eggs were incubated at 37°C with 55-60% humidity. On day 3 of incubation, a square hole was made on the top of outer shell and 2ml albumin was removed by a 0.8mm hypodermic needle to allow the detachment of the CAM development and then, the hole was carefully sealed. The eggs were returned to the incubator and the incubation was continued for 4 days. On the 7 day of incubation, blank discs with diluted sample were placed on the top of the CAM under sterile condition. The numbers of newly formed blood vessels in CAM of each treatment group were counted on a optical microscope and the neo-vascular zones of CAM were photographed. After 7 days of placing the drug sample, we checked whether the new blood vessels formed or not.

STATISTICAL ANALYSIS

Experimental data are expressed as Mean+Standard Error of Mean (SEM). Standard analysis was performed by one way ANOVA followed by Dunnet's method of multiple comparisons was employed using GraphpadInstat 3.0 software. Data were considered significant at $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$.

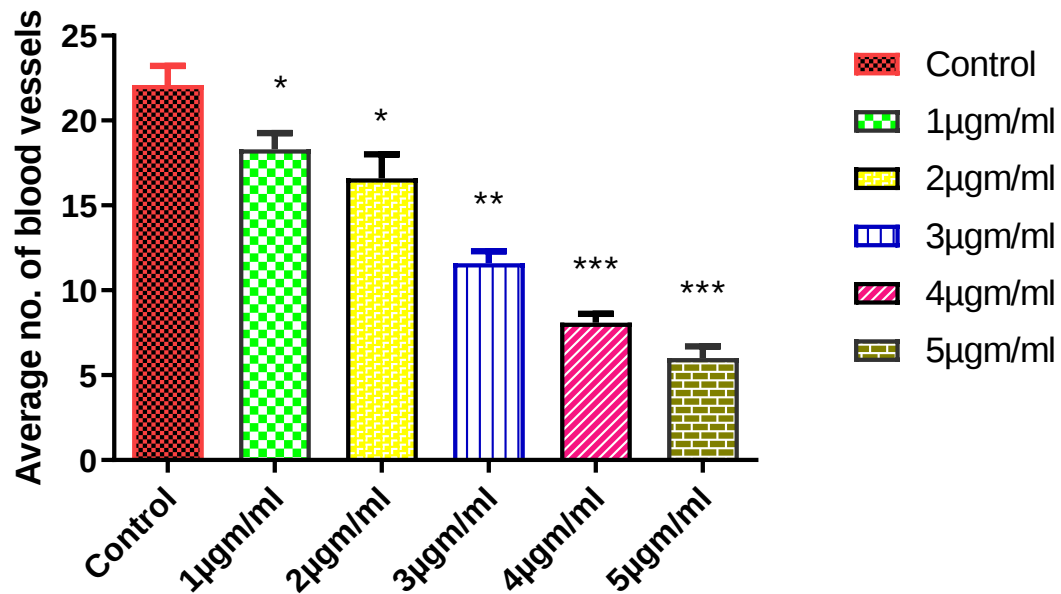
RESULT

The fruit pineapple were powdered and made into extract by using 500ml of acetone by using Maceration for 76 hrs and concentrated. And then it was evaporated and used for further studies. The evaporated sample was screening for its anti-angiogenic activity in CAM model and the values are shown below.

Table-1

Drug concentrations ($\mu\text{gm/ml}$)	Average No. of Blood vessels
Control	22.1 ± 1.12
1	18.3 ± 0.96
2	16.6 ± 1.4
3	11.6 ± 0.7
4	8.1 ± 0.52
5	6 ± 0.7

Figure: Anti-angiogenic activity of extract of *Ananas comosus*:



The results were shown :

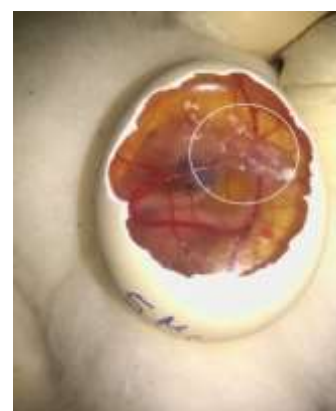
Fig -14: Anti-angiogenesis activity of the extract the fleshy part of *Ananas comosus*.



Control



1 µg/ml

2 $\mu\text{g/ml}$ 3 $\mu\text{g/ml}$ 4 $\mu\text{g/ml}$ 5 $\mu\text{g/ml}$

DISCUSSION

According to studies, Pineapple has been related cancer from occurring. It helps to prevent throat, mouth, Breast cancer as it is rich in flavonoids, bromelain, beta –carotene, vitamin A and antioxidants. CAM assay is selected for the screening of the activity. The diluted sample were tested at the dose of 1,2,3,4,5 $\mu\text{g/ml}$ because it is rich in concentration . In trial and error higher than 5 $\mu\text{g/ml}$ could collapsed the fertilized chicken embryo. Angiogenesis -Blood vessel formation. Tumour angiogenesis is the growth of new blood vessels that tumours need to grow. This process is caused by the release of chemicals by the tumour and by host cells near the tumour. The angiogenic switch can be triggered either by additional genetic alterations of tumour cells, leading to increased proliferation and hypoxia or expression of pro-angiogenic factors, or by tumour-associated inflammation and recruitment of immune cells. It plays a crucial role in the pathogenesis of various disease including rheumatoid arthritis, diabetic retinopathy, psoriasis, juvenile hemangioma and tumour growth and metastasis²⁰. Angiogenesis in inflammatory disease may also contribute to tissue growth, disordered tissue perfusion,

abnormal ossification, and enhanced responses to normal or pathological stimuli. Angiogenesis inhibitors may reduce inflammation and may also help to restore appropriate tissue structure and function. Tumour angiogenesis is the consequence of an angiogenic imbalance in which proangiogenic factors predominate over anti-angiogenic factors. Angiogenesis is the process by which new blood vessels grow. In the adult, except for a few physiological processes such as menses, wound healing and placental formation, all angiogenic processes are pathologic^{21,22}. By blocking the development of new blood vessels, one hopes to cut off the tumours supply oxygen and nutrients and therefore, its growth and spread other parts of the body [22, 23, 65]. Various animal model systems were developed, ranging from fish to mice, to study the process of angiogenesis and to observe the effects of pro-angiogenic and anti-angiogenic factors [55-56, 66].

The chorio allantoic membrane (CAM) assay has become the method of choice [27]. The CAM Model represents one of the most powerful and popular tools for analysing the angiogenic potential of purified factors and intact cells. The CAM is formed by the fusion of the mesodermal layer of two developmental structures the allantois and the chorion of the avian embryo. Being an immune-compromised but a highly vascularised tissue, the CAM serves as an indicator of the anti-angiogenic or pro-angiogenic properties of test compounds [26].

Among the five different concentrations 5µg/ml shown significant reduction (6 ± 0.7) in the formation of new blood vessels in the fertilized chicken embryo, when compared with control (22.1 ± 1.12). The extract shows some significant anti-angiogenic activity because of the presence of the bromelain (*Ananas comosus*) which were the potent anti-cancer drug. Most of the studies were done on the same plant with different solvent and extract but this acetonetic extract further on diluted sample was not performed that is the reason the plant and solvent was selected.

CONCLUSION

It is concluded that acetonetic extract of *Ananas comosus* fruit possesses significant antiangiogenic activity, and the active component which is present is bromelain is used as an anticancer agent. Further, study necessary to identify and isolate the possible active phytoconstituents responsible for the angiogenesis activity.

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