Angiogenic effect of Talisai (*Terminalia catappa* L.) crude extract on chorioallantoic membrane of chick embryo

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Abstract - Cancer is a disease with different forms affecting people of all ages worldwide. New blood vessels are important for the proliferation and growth of cancer cells. These new vessels develop from pre-existing ones, a phenomenon called angiogenesis. Terminalia catappa L. (Talisai) is said to contain the flavonoid kaempferol, which is capable of inhibiting angiogenesis. Various tests on angiogenesis have been done on the chorioallantoic membrane (CAM) of eggs because of its simplicity, low cost, and less disturbance of the cells. The leaf extract was obtained using ethanol and was proven to contain flavonoids, tannins and triterpenoids by phytochemical screening. Four concentrations (50ppm, 100ppm, 200ppm and 300ppm) of the crude Talisai leaf extract were administered to ten-day old fertilized eggs. Upon harvesting the CAM from the incubated eggs, the amount of small blood vessels was recorded by counting the number of branch points per blood vessels. Results showed that there were 60 small blood vessels counted at 50ppm, and 46, 37, and 12 at 100ppm, 200ppm and 300ppm, respectively which show a significant difference. Reduction of the small blood vessels increases as the concentration of Talisai leaf extract increases. Using a one way analysis test, a significant difference on angiogenesis using different concentrations was found as between 50ppm, 200ppm, and 300ppm. The study recommends the use of lower concentrations to show when the angiogenic inhibiting activity of Talisai starts and identify the active component responsible for its angiogenic inhibiting activity.

Keywords - angiogenesis, chorioallantoic membrane, Terminalia catappa, flavonoids

INTRODUCTION

Through the years, cancer has always been one of the leading causes of death worldwide. These statistics are based on Global Cancer Statistics 2008, the standard set of worldwide estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer (IARC) for 2008. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in females worldwide, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths. About half the breast cancer cases and 60% of the deaths are estimated to occur in economically developing countries. Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred. Lung cancer is the most commonly diagnosed cancer as well as the leading cause of cancer

death in males globally. Among females, it was the fourth most commonly diagnosed cancer and the second leading cause of cancer death. Lung cancer accounts for 13% (1.6 million) of the total cases and 18% (1.4 million) of the deaths (Bray et al., 2011).

For several thousand years, plants have been used in traditional medicine as for treating the spiritual origins of disease as well as the physical symptoms. The vast knowledge of these medicinal plants provides leads toward therapeutic concepts, accelerating drug discovery (Cheikhyoussef, Mapaure & Shapi, 2011).

Terminalia catappa is a Combretaceous plant whose leaves are widely used as a folk medicine in Southeast Asia (Annapoorani & Saroja, 2011). Also known as Tropical almond, Indian almond or Talisai, it is a large, spreading tree now distributed throughout the tropics in coastal environments. Tropical almond has a vast natural distribution in near-coastal areas of the Indian Ocean, through tropical Asia, and into the Pacific Ocean (Evans et al., 2006). Medical studies have reported that the extract of Talisai leaves and fruits have anticancer effects and that the leaf extracts can inhibit Lewis lung carcinoma cells that contribute to lung cancer (Annapoorani & Saroja, 2011). Combretaceous plants like Talisai are rich in polyphenolic compounds. Consumption of fruits, vegetables and plants rich in polyphenols is associated with a reduced risk of certain cancers, cardiovascular diseases, atherosclerosis, diabetes, and Alzheimer's disease (Annegowda et al., 2010).

Angiogenesis is the formation of new blood vessels from existing ones (Bonifacio et al., 2010; Gupta et al., 2011). It takes place during embryonic development, inflammation and angiogenesis-dependent diseases like cancer (Bonifacio et al., 2010). Tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen, and removing waste products (Mentlein & Schindler, 2006). Formation of new blood vessels accommodates the growth and spread of the cancer cells to the other parts of the body, thus resulting in the pathogenesis of the cancer itself. It paves a new way for the cancer cells to reach distant parts of the body. It is an important phenomenon in physiological situations such as embryonic development and wound healing as well as pathologic conditions like diabetic retinopathy, rheumatoid arthritis, tumor progression and atherosclerosis (Badimon, Krupinski & Slevin, 2009). Averting angiogenesis may interrupt the malignancy progression of neoplastic cells (Brown et al., 2008), treat malignant neuroblastoma (Banik et al., 2011) and prevent the emergence of different disorders it can cause, like excessive vessel growth e.g. diabetic nephropathy, psoriasis, and arthritis (Veeramani & Veni, 2010). Nowadays, antiangiogenic therapy is considered as the fourth cancer treatment besides surgery, chemotherapy and radiotherapy (Boosani & Sudhakar, 2011).

Chorioallantoic membrane is the major respiratory organ of the chick embryo that is formed by the fusion of three layers: chorionic ectoderm, allantoic

endoderm and mesoderm (Heng et al., 2008). In this research, the CAM model was used to mimic the in vivo angiogenesis of the human body. The various advantages of this model include low cost, reliability, and easy control over the model (Kreutzer, Moussy & Valdez, 2002; Heng et al., 2008; Ribatti, 2010). The traditional shell chicken chorioallantoic membrane (CAM) model has been used extensively in cancer research to study tumor growth and angiogenesis (Abe et al, n.d.; Ribatti, 2008; Berns et al., 2010). Less complicated tissue, blood sampling and direct and continuous visualization of the implant site can also be done. Its environment is more stable, flat, and has a large working area and wider field of view, excellent for imaging and longitudinal studies (Berns et al., 2010). Different chick embryo model systems allow for comprehensive analysis of specific stages and aspects of cancer cell dissemination such as tumor cell colonization in the spontaneous metastasis model, tumor cell colonization in the experimental metastasis model or tumor-induced angiogenesis in the collagen onplant model (Deryugina & Quigley, 2008).

Talisai leaves were evaluated to determine their ability to inhibit the formation of new blood vessels. Studies have shown that leaf extracts of Terminalia catappa have potential to prevent metastasis (Agbaji et al., 2011). The phytochemicals of this plant include tannins, flavonoids, and triterpenoids (Annapoorani & Saroja, 2011). The leaves of Talisai contain the flavonoid kaempferol (Joshi, Kamat & Kamat, 2008; Chikezie, 2011). Kaempferol (3,5,7-trihydroxy-2-(4- hydroxyphenyl) - 4H-1- benzopyran-4-one), one of the flavonoids present in it is also found in many edible plants and in plants or botanical products commonly used in traditional medicine. Some epidemiological studies have found a positive association between the consumption of foods containing kaempferol and a reduced risk of developing several disorders such as cancer and cardiovascular diseases (Guerrero et al., 2011). Kaempferol reduces vascular endothelial growth factor (VEGF) gene expression at mRNA and protein levels (Chen et al., 2009). Two plants of the same genus, namely Terminalia arjuna and Terminalia chebularet, also possess a certain but different flavonoid, luteolin. It has been learned that this flavonoid has an ability to inhibit angiogenesis in the murine xenograft model (Lázaro, 2009). In this regard, this study aimed to assess and evaluate the angiogenic activity of Talisai leaf extract on CAM of chick embryo given that this and some species of the same genus have phytochemicals that can inhibit angiogenesis.

It is hypothesized that the phytochemicals present in Talisai will exhibit an angiogenic effect.

MATERIALS AND METHOD

Collection of plant sample

The leaves of Talisai tree were collected from the Lyceum of the Philippines University Batangas with a required weight of approximately five hundred (500) grams. The petiole and midrib were removed. Taxonomic

identification of the plant was done by botanists of the Herbarium in the University of the Philippines, Los Baños.

Collection and incubation of eggs

One hundred twenty-two (122) fertilized white leghorn chicken eggs (Gallus domesticus) purchased in Indang, Cavite were incubated for ten (10) days before administration of Talisai leaf extract.

Preparation of Talisai leaf extract

Approximately 500 grams of fresh Talisai leaves were collected and washed with distilled water to eliminate dirt and unnecessary microorganisms, and air dried. The dried and washed leaves were cut into small pieces using an osterizer and were then immersed in 85% ethanol. They were kept in a cool, dark place for 48 hours with occasional agitation. The mixture was filtered and the filtrates were exposed under a laminar flow until almost dry (Guevarra, 2005).

Phytochemical Screening

Test for flavonoids: On one ml of the extract, a few drops of sodium hydroxide was added. Production of an intense yellow color in the plant extract, which become colorless upon addition of a few drops of dilute acid, indicates the presence of flavonoids.

Test for tannins: A few drops of 1% lead acetate were added to five ml of talisai leaf extract. Formation of a yellow precipitate indicates the presence of tannins.

Test for triterpenoids: Ten mg of the extract was dissolved in 1 ml of chloroform; 1 ml of acetic anhydride was added following the addition of 2 ml of conc. H2SO4. Formation of a reddish violet color indicates the presence of triterpenoids.

Preparation of the different concentration of the leaf crude extract

The concentrations T1 (50 ppm), T2 (100ppm), T3 (200 ppm) and T4 (300 ppm) were prepared from the obtained crude leaf extract of Talisai were calculated using a formula derived from the conversion factor (1 ppm = 1mg/L) (Mohanty & Sujana, 2010), converting milligrams of extract to grams and liters to 100 milliliters of distilled water.

Administration of Talisai Leaf Extract (Billiones, Fazar & Kashim, 2008)

The 10-day-old fertilized eggs were punctured at the blunt end having the air sac exposed for injection with a sterile syringe, making sure the CAM were not damaged. 0.3 mL of different concentrations of Talisai leaf extract was administered into the CAM, which would make the vascular network of CAM conspicuous against the white background of the egg, with a 1cc syringe fitted with a 26-gauge needle. To avoid error, one syringe was used for each

concentration. The inoculated site was covered with wax paper and sealed with a melted candle to prevent rotting and contamination. Upon administration of extract, the developing chick embryos were incubated at 37˚C for two (2) days.

Data gathering and statistical analysis

After the incubation of the eggs, the inoculated CAM was observed, counted and compared. Under a light microscope, the number of small blood vessels was recorded by counting the number of branch points per blood vessel. Branch points or points of intersection with pre-existing capillaries were also counted (Hipolito & Reyes, 2006).

The statistical tool that was used to determine the difference in angiogenesis between different concentrations is one-way analysis of variance. The Tukey method was also applied to correlate individual treatment means. All statistical analyses were done at 5% probability level (Paraguya & Remedio, 2008).

RESULTS AND DISCUSSION

Preparation of Talisai Leaf Extract

From 500 grams of the fresh crushed Talisai leaves, 15 grams of extract (Figure 1) were obtained upon evaporation under the laminar flow hood, a percentage yield of 3%.

Figure 1
Talisai leaf extract

Phytochemical Screening

The phytochemical screening of the crude extract of Talisai leaves used in this study revealed that the crude leaf extract contained tannins, triterpenoids and flavonoids as shown in Figure 2.

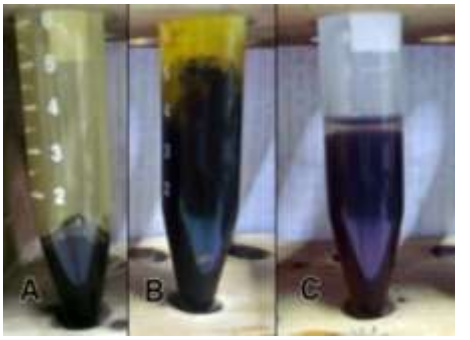

Figure 2
Phytochemical Screening : (A) Flavonoids(B) Tannins (C) Triterpenoids

Table 1 shows that the number of small blood vessels observed at different Talisai leaf extract concentrations under the microscope. At 50 ppm concentration of the Talisai leaf extract 60 small blood vessels were counted. At 100 ppm, 200 ppm and 300 ppm, there were 46, 37 and 12 small blood vessels, respectively, counted. This shows that reduction in small blood vessels increases as the concentration of Talisai leaf extract increases as depicted in Figure 3.

Table 1
Number of Small Blood Vessels Observed at Different Talisai Leaf Concentrations

Concentration of the Talisai leaf extract	Average blood vessels formed
Negative	71
50 ppm	60
100 ppm	46
200 ppm	37
300 ppm	12

Figure 3
CAM at different concentrations of the Talisai leaf extract
Negative control 50 ppm 100 ppm 200 ppm 300 ppm

Table 2 demonstrates the difference in concentrations of Talisai leaf extract as compared to the negative control. At a concentration of 50 ppm, there is a significant reduction in number of blood vessels formed as compared to the negative control. There is also a significant difference between the concentrations 100 ppm, 200 ppm and 300 ppm as compared to the negative control. This is in conformity with the study of Haddad & Vedar (2009) but using a different plant material. They revealed that at 1%, 2% and 3% of Syzigium samarangense (Macopa) crude bark extract, there was asignificant decrease in the number of small blood vessels compared to the negative control.

Table 2
Difference on the Concentration of Talisai Leaf Extract as Compared to the Negative Control

Conc	Negative Control = 71		
	T	p-value	Interpretation
50 ppm	-7.534	.000	Significant
100 ppm	-18.045	.000	Significant
200 ppm	-20.599	.000	Significant
300 ppm	-55.538	.000	Significant

Table 3 presents the differences in the inhibitory activity of each of the four (4) concentrations on the growth of the blood vessels. At 50 ppm, there is no significant difference as compared to 100 ppm but there is a significant difference at 200 ppm and 300 ppm. At 100 ppm, there is no significant difference on all concentrations. At 200 ppm, there is a significant difference as compared to 50 ppm while there is no difference between 100 ppm and 300 ppm. At 300 ppm, there is no significance between 100 ppm and 200 ppm while there is a significant difference as compared to 50 ppm. Using one way analysis of the test results, it was found out that there is a significant difference in the angiogenesis using different concentrations between 50 ppm, 200 ppm and 300 ppm since the p-value obtained is less than the 0.05 level of significance. Since there is a narrow range between 50 ppm and 100 ppm, there is only a close discrepancy of the reduction of the blood vessels that is statistically insignificant. The same p-value is seen as between 100 ppm, 200 ppm and 300 ppm.

Table 3
Multiple Comparison of the Difference of Angiogenesis Between Different Concentrations

Conc	Conc	p-value	Interpretation
50 ppm	100 ppm	.196	Not Significant
	200 ppm	.010	Significant
	300 ppm	.003	Significant
100 ppm	50 ppm	.196	Not Significant
	200 ppm	.563	Not Significant
	300 ppm	.307	Not Significant
200 ppm	50 ppm	.010	Significant
	100 ppm	.563	Not Significant
	300 ppm	.968	Not Significant
300 ppm	50 ppm	.003	Significant
	100 ppm	.307	Not Significant
	200 ppm	.968	Not Significant

CONCLUSION

The results of the study show that Talisai leaf extract has angiogenic inhibitory activity on chorioallantoic membrane of chick embryo at concentrations 50 ppm, 100 ppm, 200 ppm, and 300 ppm with a significant difference in angiogenesis between 50ppm, 200ppm, and 300ppm Reduction of the small blood vessels increases as the concentration of Talisai leaf extract increases.

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