

## **Evaluation of the Angiosuppressive Activity of *Tinospora rumphii* Boerl. Stem Extract Using the Chorioallantoic Membrane Assay in *Anas platyrhynchos* Embryos**

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### **Abstract**

Every aspect of tumor growth required vascular growth. Many natural products inhibited angiogenesis. The makabuhay plant, *Tinospora rumphii* Boerl was an important species of Menispermaceae family with common utilization in folk medicine in the Philippines. This study aimed to evaluate the angiosuppressive activity of *Tinospora rumphii* Boerl. stem extract using the chorioallantoic membrane assay in duck's (*Anas platyrhynchos*) embryos. There were thirty pieces of one day old fertile duck eggs used in the study. The setups were composed of a positive control (Retinoic acid), negative control (95% ethanol) and makabuhay stem extracts in 25%, 50%, 75% and 100% concentrations. The different concentrations of the extracts were applied on the tenth day of incubation. The antiangiogenic effect of *T. rumphii* Boerl stem extract was evaluated by taking the average number of branch points using the chorioallantoic membrane. This was determined on the 12<sup>th</sup> day of incubation. One-way analysis of variance and Scheffe test for significant antiangiogenic activity and pair-wise comparison among the treatments at 0.05 alpha level were determined respectively. Results showed that angiogenesis was induced in the negative control while angiogenesis inhibition was significantly reduced in the treatment of the stem extracts. It was supported by the statistical analysis that there was a significant difference on the antiangiogenic effect of makabuhay stem extract using the CAM assay on the vascularization of duck embryos. A significant difference was indicated between the negative control and among the treatments used. It showed that the greater the dosage, the lesser the branch points observed and counted. Pure makabuhay stem extract had the lowest branch points mean value while the 25% concentration had the highest mean value among the treatments used. Thus, these findings of the study indicated that *T. rumphii* Boerl. stem extract might had a promising antiangiogenic potential.

**Keywords:** Antiangiogenic, chorioallantoic membrane, *Tinospora rumphii* Boerl., stem extract

## **1. Introduction**

### **1.1 Background of the Study**

One The Philippines has diverse array of plant resources, yet a number of scientists feel that these resources are not fully utilized in the search for new antiangiogenic agents that would interfere with various steps of angiogenesis implicated in various pathologies such as cancer, chronic inflammation or atherosclerosis [13]. There are approximately 9,000 species

of flowering plants in the country and only about 10% are being used in the treatment of various illnesses [11, 1]. Hence, considering the many plant-derived drugs that achieve commercial sales worldwide, our country too, will improve economically once we become self-sufficient in drugs [13]. Moreover, discovery of new drugs may provide new opportunities and avenues in the field of cancer therapy.

It is well-known fact that people benefit from natural plants as medicine. With use of medicinal plants, investigations have been performed all over the world in order to find more productive and economical medicines. Medications used to cure disorders require continuous change to improve their effectiveness. With this purpose, many studies have been made comprising antioxidant and antimicrobial activities and determining other effective agents [2, 17, 21].

*Tinospora rumphii* Boerl locally known as manunggal in Bisaya is found in and nearby towns in thickets, in most or all islands of the Philippines. It is a climbing; dioeciously vine reaching a height of 4 to 10 m. The stems are up to 1 cm thick and somewhat fleshy, with scattered protuberances [16]. A decoction of the stem is considered an effective cure if used as a wash for tropical ulcers. The decoction of the stem is also an excellent vulnerary for itches, ordinary and cancerous wounds.

Although in recent years, immense progress has been made in our understanding of molecular mechanisms and cellular regulation of angiogenesis in important diseases like cancer, clinical development of antiangiogenic agents for the therapy of cancer remains challenging. Since solid tumors account for more than 85% of cancer mortality in humans, tumor growth and metastasis are dependent on blood vessels [8]. Therefore, nowadays targeting tumor angiogenesis is one of the most widely studied areas to find new therapeutic strategies. In screening potential drug candidates against angiogenesis, a broad range of plant products were screened for antiangiogenic effects [5, 12, 10, 14].

Angiogenesis is a complex biological process that occurs normally in development, turnover and remodeling of mature vascular networks [5, 12]. Angiogenesis is the formation of new vessels by endothelial sprouting, that is endothelial cell migration, proliferation and tube formation [8]. Angiogenesis is useful in some cases such as tissue infarcts when oxygen necessity increases; conversely in some cases may be harmful [18, 22]. By the surrounding neoplastic cells increasing excessively and without control, it may cause the tumor to be nourished and oxygenated and thus, encourage the growth of the tumor [8].

## 1.2 Statement of the Problem

The practice of folk medicine has become worldwide in combating diseases. Herbal therapy included *Tinospora rumphii* Boerl. in the list of plants being widely used for their potential medicinal properties. However, scientific studies to rationalize the folkloric claims regarding its cancer chemotherapeutic potential are rather scanty. The problem in determining the antiangiogenic capability of *T. rumphii* ethanol extract which would eventually provide productive leads in the discovery of novel compounds for the treatment of cancer will be investigated.

## 1.3 General Objective

This study generally aims to evaluate the angiosuppressive property of the crude ethanol extract from *T. rumphii* through duck chorioallantoic membrane assay.

## 1.4 Specific Objectives

1. To examine the effects of the different concentrations of *T. rumphii* extract on the percent vascularity of the chorioallantoic membrane.
2. To determine if there is a significant difference on the percent vascularity among the concentrations used.

## 1.5 Significance of the Study

It is well-known fact that people benefit from natural plants as medicine. With use of medicinal plants, investigations have been performed all over the world in order to find more productive and economical medicines. Medications used to cure disorders require continuous changes to improve their effectiveness. This continuous search for new bioactive compounds from plants fuels the interests of natural product chemists, biologists and clinicians to produce more effective but less toxic drugs.

## 2. Methodology

**2.1 Plant Material.** Preliminary identification of the plant material was based on De Tavera (1901), Madulid (2000), Quisumbing (1978), Stuart (2000) and voucher specimen which was deposited before at the University of the Philippines Los Banos Herbarium under Dr. Inocencio Buot Jr. The plant material was further confirmed by Dr. Gerard Penecilla, a plant systematics professor at West Visayas State University. The fresh stems of *Tinospora rumphii* Boerl. (manunggal) were collected in Barangay Parara Sur, Tigbauan, Iloilo on January 25, 2012.

**2.2. Filter Paper Disc Preparation.** Filter paper was punched with a 2-holed puncher to form the paper discs (approximately 5 mm in diameter). The paper discs were sterilized by autoclaving for 15 minutes.

**2.3. Extract Preparation.** Air-dried and chopped stems of 200 grams manunggal stems was soaked in 95% ethanol for 48 hours. The soaked solution was filtered using Whatmann filter paper. The filtrate in ethanol rotary evaporated at West Visayas State University to a small volume. The concentrated extract was partitioned to 25%, 50%, 75% and 100% concentrations as the experimental group. 90% ethanol and retin A cream as source of retinoic acid were used as the negative and positive control respectively.

**2.4 Assay Proper.** The 1-day old fertilized duck eggs were cleaned with 70% ethanol. The eggs were then incubated for 10 days at 37<sup>0</sup>C and 70% humidity. A window in the egg shell about 1x1 cm was made to expose the CAM to direct access for experimental manipulation. The test plant extract was absorbed on the filter paper disc. About two milliliter of the fluid inside the egg was extracted. Then, the treated filter paper disc was placed directly onto the CAM. The treated eggs were sealed with plastic tape and were incubated for two days. Day 10 is the subject for experimental treatment because between day 8 and day 10, the developing CAM vasculature is ready to sprout in response to additional proangiogenic stimuli and, in turn, is very responsive to antiangiogenic factors [3]. On the 12<sup>th</sup> day of incubation, the CAMs were harvested by removing the hard shell leaving intact the soft membrane covering the embryo. The CAM was examined for the number of blood vessel branch points.

**2.5 Sampling Treatment in a Completely Randomized Design (CRD).** A 6 x 5 tray was numbered 1 to 30. In one bowl, 30 rolled papers numbered 1 to 30 were placed. In another bowl, 30 rolled papers five each labeled negative control, positive control, 25%, 50%, 75% and 100%. For every egg picked, it was placed in the location indicated by the number and treatment assigned by the number on the tray. It was repeated without replacement until all eggs were assigned to the designated tray label.

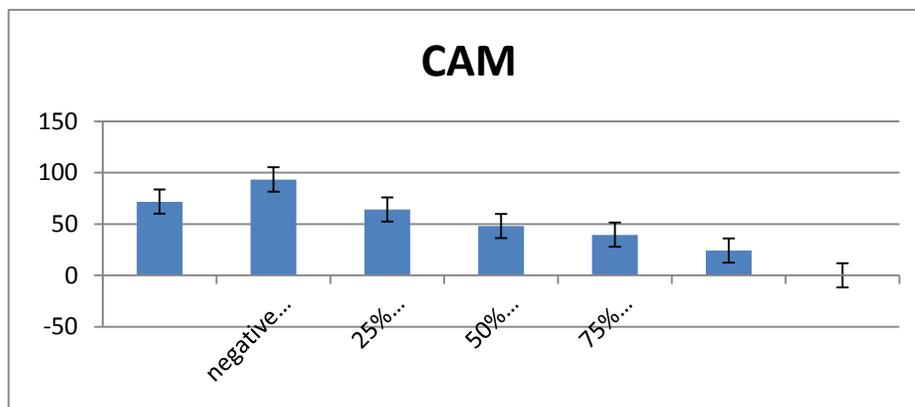
**2.6 Visual Assessment and Photography.** The CAM at the site of application for angiogenesis was examined. In this assay, quantitation was performed 2-3 days after implantation and involved counting the number of CAM vessels in the area of filter paper disk [3]. In response to proangiogenic stimuli, the newly formed blood vessels appear converging toward the disk in a wheel-spoke pattern. Inhibition of angiogenesis by antiangiogenic compounds results in the lack of new blood vessel formation and sometimes in disappearance of pre-existing vessel networks. Four quadrants of the CAM in the area were drawn. The blood vessel branch point at each area of the quadrant was counted manually in a clockwise direction.

**2.7 Statistical Analysis.** Comparison between groups was made by One-Way Analysis of Variance followed by Scheffe test for pairwise comparison by the aid of SPSS v. 11.5. Differences with  $P < 0.05$  between experimental groups was considered statistically significant.

### 3. Results and Discussion

#### 3.1. Results

Duck Egg Chorioallantoic Membrane Assay. Angiogenic activity of ethanolic extract of *Tinospora rumphii* Boerl. stem extract by CAM assay was determined after 12 days of incubation. It strongly elicited an antiangiogenic response as shown in Figure 1. Furthermore, it is supported in Figure 2.



**Figure 1. Antiangiogenic Activity of *Tinospora rumphii* Boerl. Stem Extract on the CAM**

Angiogenesis was induced in the negative control while angiogenesis inhibition was strongly reduced in the treatment of manunggal stem extracts. This is shown in Table 1.

**Table 1. Average Branch Points of CAM**

Treatment	Mean	Standard Deviation
Positive Control	71.80	24.01
Negative Control	93.40	8.84
25% Concentration	64.20	22.58
50% Concentration	48.00	25.92
75% Concentration	39.60	12.34
100% Concentration	24.20	6.09



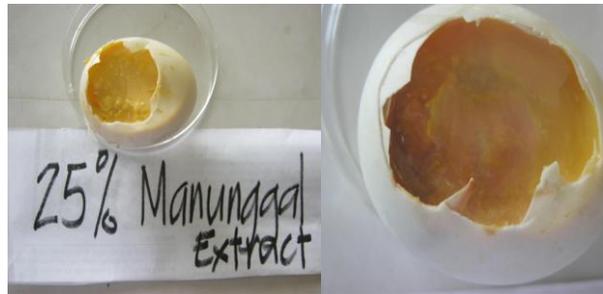
**Figure 2a. CAM in 100% Manunggal Extract**



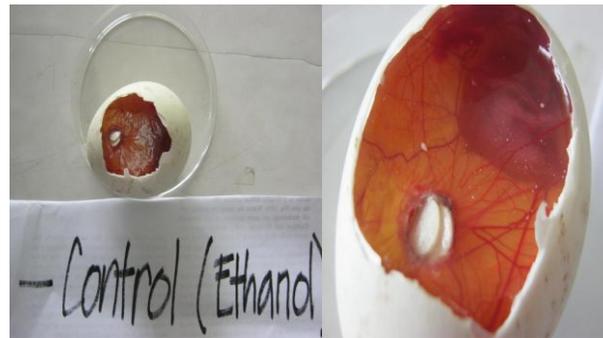
**Figure 2b. CAM 75% Manunggal Extract**



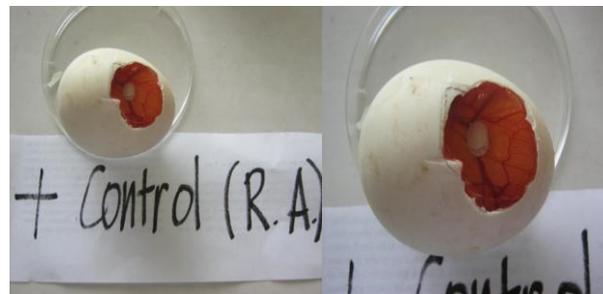
**Figure 2c. CAM in 50% Manunggal Extract**



**Figure 2d. CAM in 25% Manunggal extract**



**Figure 2e. CAM in the Negative Control**



**Figure 2f. CAM in the Positive Control**

**Figure 2. Photographs of CAM in the Different Treatments**

### 3.2 Statistical Data Analysis

Statistical comparisons between the control and treated groups were carried out using One-way analysis of variance. The difference between groups for pairwise comparison was assessed using Scheffe test.

There is a significant difference in the antiangiogenic effect of manunggal stem extract using the CAM assay on the vascularization of duck embryos  $F\text{-value}=9.045$ ,  $df=5$ ,  $0.000 < 0.05$ . Statistical result showed that the CAM treated with manunggal extract at various concentrations has antiangiogenic effect. It indicates that the greater the dosage, the lesser the branch points that were observed and counted.

Furthermore, this is supported using the Scheffe test for pair-wise comparison in Table 4. There is a significant difference between the positive and 100% concentration; between the negative control and 50%, 75% and 100% concentrations; between the negative control and 50%, 75% and 100% concentrations; between 50% and negative control; between 75% and the negative control; and between the 100% concentration and the positive and negative controls.

### 3.3 Discussion

Over the recent years, more attention has been focused on the anti-angiogenic and anti-tumor effects of non-toxic compounds from natural products. Angiogenesis mainly depends on proper activation, proliferation, adhesion, migration and maturation of endothelial cells [9]. Inhibition of angiogenesis has been considered to be advantageous for prevention of tumor growth and metastatic activity [9]. Some antiangiogenic substances were identified to be effective in animal models of arthritis and several antirheumatic drugs such as methotrexate, contain antiangiogenic activity [7]. Some plant extracts contain many active ingredients. They are complex chemical cocktails with medicinal properties that affect tumor angiogenesis [9]. A wide range of plants that contains compounds were identified and their phytochemicals were isolated and characterized [6]. The chick chorioallantoic membrane assay was used for examining the antiangiogenic activity of *T. rumphii* Boerl. The results indicated that *T. rumphii* Boerl. in a dose dependent manner inhibits angiogenesis *in ovo*.

In this direction, the plant is being actively explored as a source of new chemical substance that can inhibit angiogenesis. Independent of this effect in this study, it is clearly elucidated that antiangiogenic activity of *Tinospora rumphii* Boerl. stem extract by performing *in ovo* antiangiogenesis assay. It has been observed that manunggal stem extract significantly inhibits the development of capillary networks in CAM. The observation in this study suggests that *T. rumphii* Boerl. stem extract exhibits a strong antiangiogenic activity. It may have the potential to be a useful deactivator of numerous serious diseases characterized by regulated angiogenesis.

### 4. Conclusions and Recommendations

The result of this study shows the use of *Tinospora rumphii* Boerl. stem extract that has a potential of antiangiogenic property. The average branch points in the duck CAM was decreased in a dose-dependent manner. Thus from the macroscopic observation, it has been concluded that *T. rumphii* Boerl. stem extract has a promising antiangiogenic agent and a possible source of chemotherapeutic agent against tumors. Further study is required to define more precisely the mechanism involved by which *T. rumphii* Boerl stem extract inhibits vascularization in the duck CAM *in ovo* as well as the pathological relevance of these findings.

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## References

- [1] Bernas G, Gonzales ER, Solevilla RC and Ysrael MC, "Pharmacology-toxicology section: a guidebook to plant screening: phytochemical and biological", University of Santo Tomas: Publishing House, Espana, Manila, (2005).
- [2] Candan F, et. al., "Antioxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium subsp. Millefolium Afan.(Asteraceae)*", J. Ethnopharmacol, vol. 87, (2003), pp. 215-220.
- [3] Deryugina EI and Quigley J, "Chick embryo chorioallantoic membrane models to quantify angiogenesis induced by inflammatory and tumor cells or purified effector molecules", Methods Enzymol., vol. 444, (2008), pp. 21-41.
- [4] de Tavera P, "The Medicinal plants of the Philippines. P. Blakiston's Son and Co., (1901).
- [5] Elluru SR, et. al., "Antiangiogenic properties of viscum album extracts are associated with endothelial cytotoxicity", Anticancer Res., vol. 29, (2009), pp. 2945-2950.
- [6] Fan TP, Yeh JC, Leung KW, Yue PYK and Wong RNS, "Angiogenesis: from plants to blood vessels", Trends Pharmacol Sci., vol. 27, no. 6, (2006), pp. 297-309.
- [7] Folkman J, "Angiogenesis", Annu Rev Med., vol. 57, (2006), pp. 1-8.
- [8] Goze I, Cetin A and Goze A, "Investigation of effects of essential oils of *Origanum minutiflorum* O Schwarz PH Davis and *Cyclotrichium niveum (Labiatae)* plants on angiogenesis in shell-less chick embryo culture", American Journal of Biotechnology, vol. 9, no. 14, (2010) April 5, pp. 2156-2160.
- [9] Keshavarz M, Bidmeshkipour A, Mostafaie A, Mansouri K and M-Motiagh HR, "Anti-tumor activity of *Salvia officinalis* is due to its antiangiogenic, antimigratory and antiproliferative effects", Cell Journal, vol. 12, no. 4, (2011) Winter, pp. 477.
- [10] Liu D, Liu Z, Woltering EA and Greenway FL, "Antiangiogenic effects of curcumin in pure versus in extract forms", Pharm Biol., vol. 46, (2008), pp. 677-682.
- [11] Madulid DA, "A pictorial cyclopedia of Philippine ornamental plants", 2<sup>nd</sup> ed. Manila: Bookmark, (2000), pp. 389.
- [12] Mojzis J, Varinska L, Mojzisoava G, Kostova I and Mirossay L, "Antiangiogenic effects of flavonoids and chalcones", Pharmacol. Res., vol. 57, (2008), pp. 259-265.
- [13] Olarte EI, "The Don Mariano Marcos Memorial State University (DMMMSU)-potential antiangiogenic property of *Cassia alata* L. hexane extract on the embryonic blood vessels of duck", *Anas domesticus* embryos, vol. 1, no. 3, ISSN No. 0119-4062, (2007) January-June.
- [14] Oner FH, Bezerra Y, Peyman GA, Conway M, Lewis JM, Liu Z, Greenway F, Woltering EA, "Antiangiogenic effect of a Chinese sweet leaf extract in experimental corneal neovascularization", Pharm. Biol., vol. 45, (2007), pp. 46-48.
- [15] Quisumbing E, "Medicinal plants of the Philippines", Manila: Katha Publishing Co., (1978), pp. 1262.
- [16] Salazar NP, Sabordo NT, Romero RC, Montalban CS, Acacio MR, Tarrayo MG, Bustos DG and Moragas TR, "*Tinospora rumphii* Boerl. (Makabuhay) in the treatment of scabies", Phil J. Microbial Infect Dis, vol. 16, no. 1, (1987), pp. 25-29.
- [17] Sokmen M, Serkedjieva J, Daferera D, Gulluce M, Polissiou M, Tepe B, Akpulat A, Sahin F and Sokmen A, "In vitro antioxidant, antimicrobial and antiviral activities of the essential oil and various extracts from herbal parts and callus cultures of *Origanum acutidens*", J. Agric. Food Chem., vol. 52, (2004), pp. 3309-3312.
- [18] Staton CA, Lewis C and Bicknell R, "Angiogenesis assay, a critical appraisal of current techniques", John Wiley and Sons LTD. The Atrium, Southern Gate, Chichester, (2006).
- [19] West Sussex PO19 85Q England, (1985), pp. 183-186.
- [20] Stuart G, "List of medicinal plants in English and Tagalog", <http://www.stuartxchange.org/CompleteList.html>, (2000).
- [21] Tepe B, Sokmen M, Sokmen A, Daferera D and Polissiou M, "Antimicrobial and antioxidative activity of the essential oil and various extracts of *Cyclotrichium organifolium* (Labill.)", Manden and Scheng. J. Food Eng., vol. 69, (2005), pp. 335-342.
- [22] Tufan AC and Tufan L, "The effect of ethanol exposure on extraembryonic vascular development in the chick area vasculosa", Cells Tissues Organs, vol. 97, (2003), pp. 175-184.

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