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Antiangiogenic Activity of *Tinospora rumphii* Boerl (Makabuhay) Leaf and Stem Extracts

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Abstract

In the Philippines, many herbal plants are being used traditionally for the treatment of common diseases and disorders. There are medicinal plants which inhibit angiogenesis or the formation of new blood vessels. One of them is the Tinospora rumphii Boerl, a large, abundantly growing vine, commonly known as "Makabuhay". It is being used by rural folks for the treatment of cancerous wounds and tropical ulcers. This study evaluated the antiangiogenic activity of T. rumphii leaf and stem extracts using the number of new blood vessels formed in the chorioallantoic membrane of fertilized mallard embryos after 48 hours of treatment. Results of the CAM assay showed that the T. rumphii extracts exhibit antiangiogenic property based on the less blood vessels formed in the chorioallantoic membrane of mallard embryos. The combination of T. rumphii leaf and stem extracts yielded the lowest mean number of new blood vessels formed as compared to the leaf extract and the stem extract. One-way ANOVA revealed that there is a significant difference in the mean number of blood vessels formed, while method of Least Significant Difference specified that the combination of T. rumphii leaf and stem extracts is significantly more antiangiogenic than the leaf extract and the stem extract. These findings led to the conclusion that the T. rumphii extracts have antiangiogenic potential and that the combination of leaf and stem extracts inhibit angiogenesis more than the leaf extract or the stem extract.

Keywords: Antiangiogenesis, *Tinospora rumphii* Boerl, leaf and stem extracts, Chorioallantoic Membrane assay.

Introduction

The use of plants to treat illnesses has been an accepted practice among indigenous people throughout the world[1-3]. In some traditional practices, extracts from different parts of a plant were used for therapeutic purposes. At present, plant extracts have been studied for their anti-inflammatory, antioxidant, antiviral, and anticancer activities[4-7]. Studies have also shown that the combination of plant extracts can have synergistic or additive effects[8].

In the Philippines, many plants have been studied for their antiangiogenic properties to provide possible treatment for cancer. One of these plants is the *Tinospora rumphii*. Its common Tagalog name is Makabuhay which means "to give life". The *T. rumphii* plant is a large, deciduous vine with extensively spreading and climbing branches and is abundantly growing in the Philippines. The aqueous crude stem extract of *T. rumphii* was not toxic on normal cell lines and moderately blocked the proliferation of selected human cancer cells[9]. A study on the crude ethanolic extract of *T. rumphii* stem showed that the number of blood vessel branch points in the chorioallantoic membrane of duck embryos was significantly reduced when treated with various concentrations of stem extracts. Specifically, there was lesser branch points counted when subjected with a greater dosage of the extract[10]. However, *T. rumphii* has been used also as abortifacient, for irregular menstruation and for late menstruation[11]. This effect is due to the quaternary alkaloid compounds and chemical constituents present in *T. rump*hii which can interfere with microtubule function[12].

Angiogenesis has become a an important issue in fighting against the progress of cancer because it was thought that antiangiogenic drugs prevent the growth of cancer cells by blocking the development of new blood vessels[13,14]. There is a challenge, therefore, to investigate plant extracts which have the potential to inhibit angiogenesis. These ideas motivated the researchers to conduct a study on the antiangiogenic property of *T. rumphii* particularly on its leaf extract, stem extract, and the combination of leaf and stem extracts.

Materials and Methods

Preparation of Materials and Extracts: Two kilograms of *T. rumphii* plant samples were collected and were botanically authenticated at the Botany Division of the National Museum, Manila. The plant samples included the *T. rumphii* leaves and stems (Fig. 1). The fresh plant samples were washed with distilled water and were separately air-dried and pulverized into coarse powder. For the combination of leaf and stem extracts, 500g of each pulverized leaf and stem were mixed together prior

to soaking[8]. The extracts were prepared from the pulverized plant samples which were soaked in 95% ethanol for three weeks, and subsequently filtered and subjected to rotary evaporation. All materials used were sterilized for two hours prior to experimentation. The pure extracts were refrigerated until used.

Chorioallantoic Membrane (CAM) Assay: Twenty-five 3-day old fertilized mallard (*Anas platyrhynchos*) eggs were washed and cleaned with 70% alcohol. The eggs were incubated horizontally for 5 days at 37^{o}C with constant humidity. Five replicates were used for each treatment. Experimentation proper was conducted under the fume hood to prevent bacteria contamination. A small opening in the egg shell, opposite to the position of the embryo, was done to expose the chorioallantoic membrane for experimental manipulation. The slightly dried, treated filter disc was placed directly into the chorioallantoic membrane. Then, the small opening of the 8-day old treated mallard eggs were tightly covered with parafilms and incubated horizontally for 2 days[15]. After incubation, the hard shell immediate to the treatment site was removed, revealing the chorioallantoic membrane surrounding and under the treated filter disc. The treatment site was documented using a camera and the branching points were counted through Adobe Photoshop CS3[10].

Statistical Tools: Mean was used to determine the average branching points of blood vessels formed in the CAM of mallard embryos. One-Way Analysis of Variance for Complete Randomized Design (ANOVA-CRD) with equal replications was used to determine if there is a significant difference in the mean count of blood vessels in the CAM of mallard embryos when treated with*T. rumphii* extracts. The Least Significant Difference (LSD) Test was used to compare treatment means and to establish which pair of means significantly differ. All tests were done at the 0.05 level of significance.

Results and Discussion

The antiangiogenic activities of *T. rumphii* leaf, stem, and the combination of leaf and stem extracts were based on the number of blood vessels formed in the chorioallantoic membrane (CAM) of mallard embryos. Table 1 shows the average number of blood vessels formed in the CAM of mallard embryos using five treatments: distilled water (negative control), methotrexate (positive control), leaf extract, stem extract, and the combination of leaf and stem extracts.

Mallard embryos treated with distilled water yielded the highest mean of 24.8 blood vessels formed (figure-2). This indicates that water, being the negative control, is the least antiangiogenic among the five treatments. The *T. rumphii* extracts, however, yielded much lower number of blood vessels. The stem extract yielded an average of 13.2 blood vessels formed (figure-3), the leaf extract yielded a mean of 11.0 blood vessels (figure-4), while the combination of *T. rumphii* leaf and stem extracts, however, yielded only an average of 4.2 blood vessels (figure-5). These results indicate that the *T. rumphii* extracts possess antiangiogenic properties. Comparative analysis of the mean blood vessels formed, however, showed that those treated with the combination of stem and leaf extract have very low mean as compared to those treated with *T. rumphii* stem extract and with *T. rumphii* leaf extract. This means the combination of *T. rumphii* leaf and stem extracts is the most antiangiogenic when compared to the individual leaf and stem extracts. The presence of flavonoids in both *T. rumphii* leaf and stem extracts contributes to its suppressing property towards angiogenesis[16]. In addition, flavonoids decrease the thickness of blood vessels and vascular endothelial growth factor (VEGF) levels[17]. The health benefits of flavonoids are usually linked to its property to inhibit xanthine oxidase and other enzymes. Flavonoids were proven to possess anti-inflammatory, antioxidant, anti-allergic, hepatoprotective, thrombolytic, antiviral, and anticarcinogenic properties[18-20].

Table-1
Antiangiogenic Activity of *T. rumphii* Extracts Based on Blood Vessels Formed

Treatment	Number of Blood Vessels in the CAM					Mean
	R_1	R_2	R_3	R_4	R_5	
Distilled Water	20	24	27	25	28	24.8
Methotrexate	0	2	2	0	2	1.2
Stem Extract	12	13	12	14	15	13.2
Leaf Extract	10	11	10	13	11	11.0
Leaf+Stem Extracts	5	3	4	4	5	4.2

Figure-1
***T. rumphii* leaves and stems**

In the CAM assay, methotrexate was used as the positive control. It yielded only an average of 1.2 blood vessels. In the first and fourth replicates, no blood vessels were formed (figure-6). This means methotrexate is the most antiangiogenic among the five treatments. Because of its high antiangiogenic properties, methotrexate is used to treat cancer of the breast, psoriasis, corneal angiogenesis, and arthritis[21]. The comparative antiangiogenic activity of *T. rumphii* extracts is presented graphically in figure-7.

To determine whether there is a significant difference in the antiangiogenic activity of the *T. rumphii* extracts, One-way ANOVA was used on the mean number of blood vessels formed in the CAM of mallard embryos. The result showed that there is a significant difference in the antiangiogenic activity of the five groups exposed to different treatments in this study. This is shown by the *F-value* of 142.520 with $p<.05$ (table-2). This implies that at least one pair of the means of blood vessels is significantly different. Thus, the null hypothesis that there is no significant difference in the mean number of blood vessels in the CAM of mallard embryos using the five treatments is rejected.

To test further which pairs of mean blood vessels are significantly different, the Least Significant Difference (LSD) Test was used. Results of the LSD Test on Table-3 showed that the combination of *T. rumphii* leaf and stem extracts is significantly more antiangiogenic than the stem extract ($p=.000$) and the leaf extract ($p=.000$). The negative mean difference of the combined leaf and stem extract with respect to stem extracts and leaf extract indicates that there are less blood vessels formed when treated with the combined leaf and stem extracts than when treated with the individual stem and leaf extracts.This suggests that *T. rumphii* leaf and stem extracts synergize their antiangiogenic activity. The *T. rumphii* leaves and stems contain quaternary alkaloid compounds like protoberberine bases berberine and palmatine with jatrorrhizine and the aporphine base magnoflorine[22]. The *T. rumphii* contains chemical constituents that haveanti-cancer properties which can interfere with microtubule function such as borapetol A, borapetol B, borapetoside A, borapetoside B, tinocrisposide, N-formylanondine, N-formylnornuciferine, N-acetyl nornuciferine, γ-sitosterol, picrotein, and tinotubride[12]. *T. rumphii* stem extract, however, showed no significant difference in antiangiogenic activity with that of the leaf extract ($p>.05$). Their mean difference is only 2.20 which is not significant at the .05 level.

Table-2
ANOVA Results on the Antiangiogenic Activity of *T. rumphii* Extracts

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	*F*	*Sig.*
Treatments	4	1687.440	421.860	142.520*	.000
Error	20	59.200	2.960		
Total	24	1746.640			

*Significant at the 0.05 level

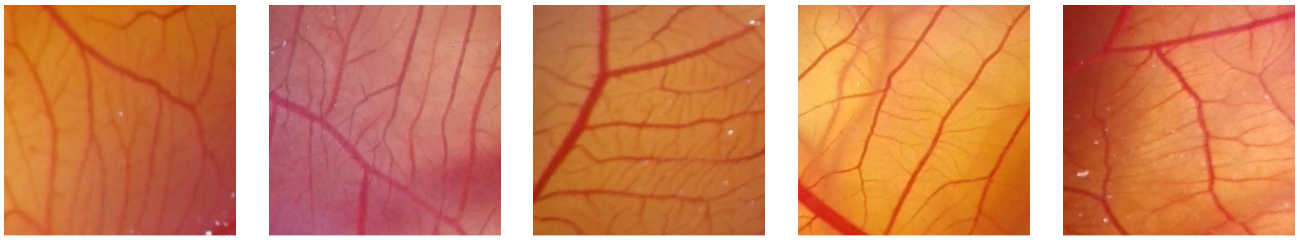

Figure-2
CAM treated with distilled water (negative control)

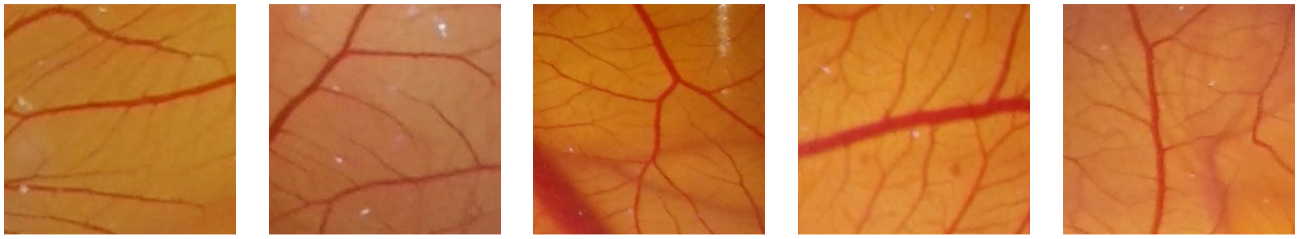

Figure-3
CAM treated with *T. rumphii* stem extract

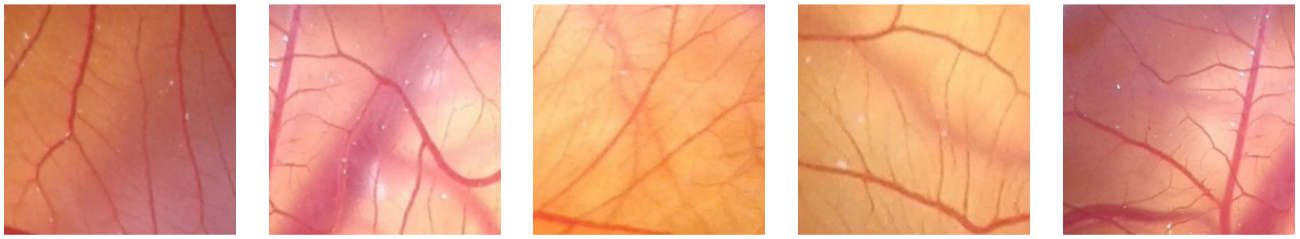

Figure-4
CAM treated with *T. rumphii* leaf extract

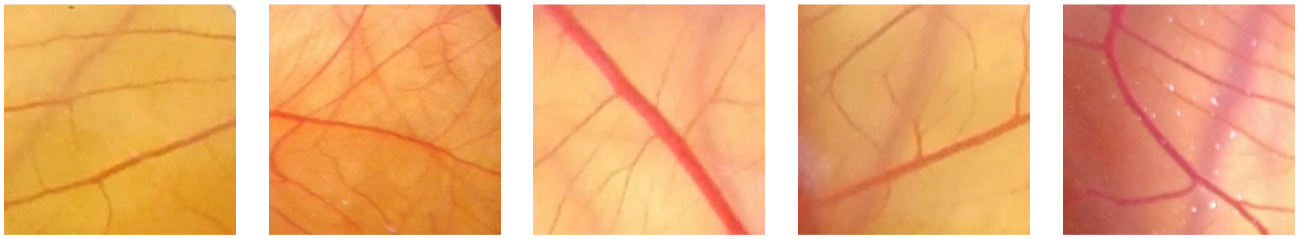

Figure-5
CAM treated with *T. rumphii* leaf+stem extract

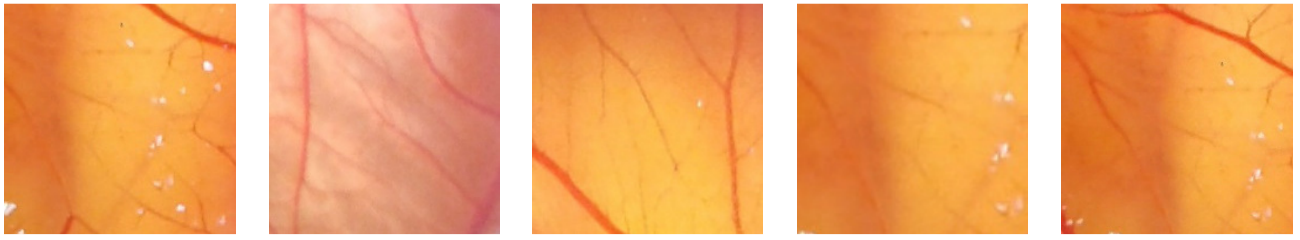

Figure-6
CAM treated with methotrexate (positive control)

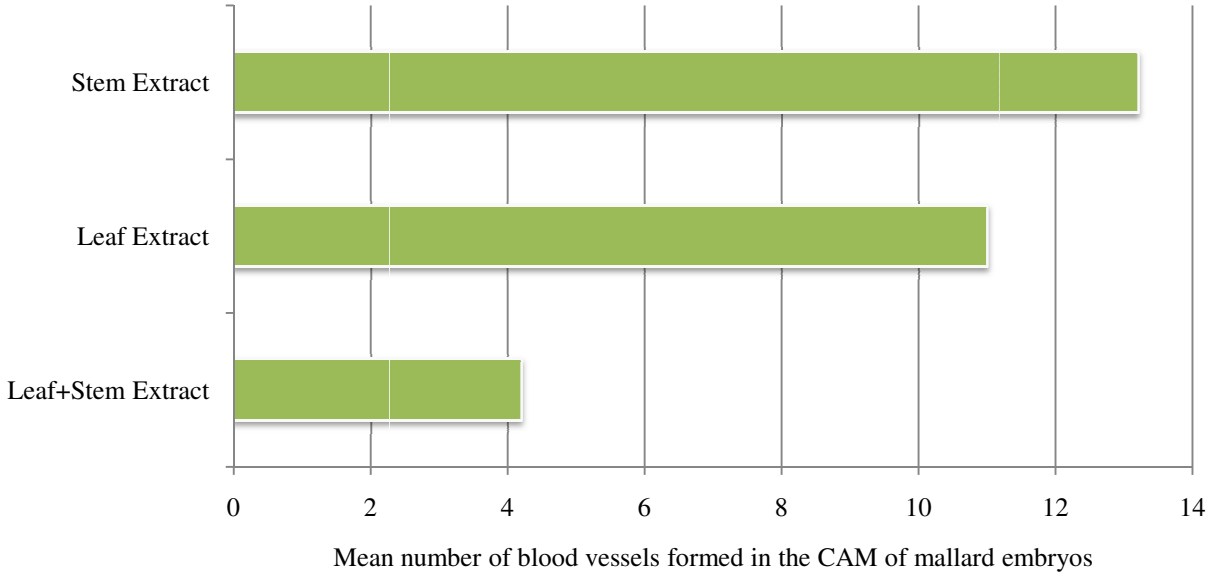

Figure-7
Mean Number of Bood Vessels Formed When Treated With *T. rumphii* Extracts

Table-3
Multiple Comparison of Mean No. of Blood Vessels Formed Using LSD Test

Treatment (I)	Treatment (J)	Mean Difference	*p-value*	Interpretation
Combination of *T. rumphii* Leaf and Stem Extracts	Distilled Water	-20.6*	.000	Significant
	Methotrexate	3.0*	.012	Significant
	Stem Extract	-9.0*	.000	Significant
	Leaf Extract	-6.8*	.000	Significant
T. rumphii Stem Extract	Distilled Water	-11.6*	.000	Significant
	Methotrexate	12.0*	.000	Significant
	Leaf Extract	2.2	.057	Not significant
	Leaf+Stem Extract	9.0*	.000	Significant
T. rumphii Leaf Extract	Distilled Water	-13.8*	.000	Significant
	Methotrexate	9.8*	.000	Significant
	Stem Extract	-2.2	.057	Not significant
	Leaf+Stem Extract	6.8*	.000	Significant

*Significant at the 0.05 level, $LSD_{.05} = 2.26$

Conclusion

Based on these macroscopic findings, it can be concluded that the *T. rumphii* plant is a potential source of antiangiogenic substances particularly the combination of its leaf and stem extracts. The mean number of blood vessels formed in the CAM of mallard embryos was significantly reduced when treated with the combined *T. rumphii* leaf and stem extracts. It is therefore recommended that further research be conducted to determine the molecular mechanism on how the extracts inhibit angiogenesis and their possible application to treat diseases involving abnormal vascular growth such as cancer.

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