Assessment of the Combination of Anthocyanin and Quercetin as an Angiogenic Inhibitor through a Chick Embryo Chorioallantoic Membrane Assay

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Abstract – Angiogenesis is a normal physiological process where new capillary branches sprout from preexisting blood vessels. Excessive angiogenesis can lead to the development of cancer, atherosclerosis, rheumatoid arthritis, etc. Anthocyanin is known for inhibiting angiogenesis in human endothelial cells. Quercetin has also shown an inhibitory effect when it was administered to the chorioallantoic membrane of chicken eggs. However, it has been proven that single flavonoids are less effective compared to when they are combined. This study aimed to assess the combined effect of anthocyanin and quercetin in inhibiting blood vessel growth in a chick embryo chorioallantoic membrane. Anthocyanin, quercetin, and their combination were each prepared with a concentration of 15 mg/L. The treatments were administered to seven-day-old chicken eggs and dimethyl sulfoxide served as the positive control. The appearance of each chrorioallantoic membrane was evaluated using the software ImageJ through calculation of fractal dimension values three days after the treatments were introduced. The values were then statistically analyzed through Kruskal-Wallis where it was found out that there was no significant difference between all treatments when compared to the controls. Of the three flavonoid treatments, the combination of anthocyanin and quercetin appeared to be the most effective in reducing blood vessel growth. This shows that the combination of anthocyanin and quercetin is more efficient in inhibiting angiogenesis. This treatment could be a novel source of treating pathological angiogenesis.

Introduction. – Angiogenesis is a normal physiological process primarily occurring in embryo development where new capillary branches made of endothelial cells sprout from preexisting blood vessels (Ucuzian et al. 2010). It occurs in the healthy body after ovulation and also for the restoration of blood flow to injured tissues to provide oxygen and nutrients for repairing cells [9]. Normally, angiogenesis is modulated by the angiogenic switch where there is a balance of pro- and antiangiogenic factors secreted by both host and tumor cells which when balanced in favor of pro-angiogenic factors lead to new vessel formation [2]. However, when this balance is disturbed, excessive or insufficient angiogenesis can occur which could lead to the pathology of various diseases such as solid cancers, coronary artery disease, stroke, diabetic blindness, and psoriasis affecting more than one billion people worldwide.

Natural remedies such as curcumin, quercetin, ginger, proanthocyanidin have been discovered to have a high degree of antiangiogenic activity. For example, the synergistic effect of turmeric tea powder made of curcumin, quercetin, vitamin E, and vitamin C suppressed angiogenesis in the chorioallantoic membrane of 10-day old duck eggs. This is shown by the decrease in blood vessel sprouting as the concentration of the powder increased [14]. Vitamin E and vitamin C are vitamins while curcumin and quercetin are flavonoids. Flavonoids are phytochemicals that give color to most flowers, fruits, and seeds. They have been abundantly studied for their inhibitory capacity in angiogenesis by: (1) prevention of capillary sprout formation and endothelial cell proliferation and migration, (2) modulation of angiogenic receptor signaling pathways like VEGF and FGF, and (3) inhibition of degradation of the basement membrane [27]. The problem is that although single flavonoids have al-

ready been proven to be effective as angiogenic inhibitors, evidence has shown that single antiangiogenic agents have limited efficacy [20]. It is suggested that synergistic activity of natural products be studied due to the range of complex organic chemicals they possess which can inhibit angiogenesis by interacting with multiple pathways and other ways that can affect cell signaling [20].

The pairing of anthocyanin and quercetin has already been proven by Hidalgo et al. (2009) to exhibit a synergistic effect in terms of antioxidant activity. However, there is no study conducted on the combination of these two bioflavonoids as an angiogenic inhibitor. Theoretically, these two flavonoids are expected to react in such a way that the chemical compositions of anthocyanin and quercetin will be retained. There will be no new compound formed, only a mixture wherein each of the two flavonoids will target the blood vessels separately using the same mechanism which is the prevention of proliferation of endothelial cells responsible for the formation of new capillaries.

Methods. –

Acquisition of materials. One hundred fifty 0-day old chicken eggs were bought from the Hatchery and Incubation Unit of West Visayas State University - Calinog. Twenty-five grams each of anthocyanin and quercetin was purchased from Sinuote Biotech and shipped from China. Other materials were bought from Mercury Drug, E. Lopez Street, Iloilo City, Berovan Marketing Inc., 100 Com Civil, JM Basa Street, Iloilo City, and Citi Hardware, Diversion Road, Iloilo City and SM Savemore Jaro, Libertad St, Jaro, Iloilo City.

Incubation of chicken eggs. All chicken eggs purchased were put inside a manual industrial egg incubator with a temperature range of 38C to 38.5C and relative humidity of 57%. The eggs were candled on the third day of incubation to check their fertility, as measured by the visible growth of blood vessels in the egg when viewed under a candler. All infertile eggs were properly disposed of after candling. During the entire incubation period, the eggs were turned to a 45- degree angle from their vertical positions six times a day for seven days to ensure balanced temperature flow throughout the eggs and prevent the blood vessels from sticking to the shell.

Preparation of flavonoid treatments. For the treatment with anthocyanin only and quercetin only, 0.0151 g [5] each of anthocyanin and quercetin was weighed on an analytical balance. The two flavonoid powders were then separately transferred to 50-mL beakers and mixed with 50 mL of dimethyl sulfoxide (DMSO) [16]. The solutions were stirred until the powders dissolved. Each solution was transferred to a one-liter beaker where it was diluted with distilled water until the 1 liter line was reached. The mixture was stirred. For anthocyanin and quercetin, 0.0076 each of anthocyanin and quercetin were combined and the same process was followed. All treatments were then transferred to reagent bottles and stored in a refrigerator for 24 hours at normal refrigeration temperature, 1.6 C.

Administration of treatments. All chicken eggs were taken out of the incubator on the seventh day and their shell surfaces were individually cleaned with 70% ethyl alcohol using a common paper towel. A slit was cut using a 12-inch hacksaw on the center of the blunt side of the egg where the air sac was located. A micropipette was then used to fill the barrel of an insulin syringe with 20 L of 15 mg/L (DMSO, 0.5%) of each treatment. The insulin syringe was inserted into the slit on each egg and positioned on the lower half of the air sac. Each treatment was then administered. The eggs were put back in the industrial egg incubator and further incubated for three days before viewing of blood vessels [21].

Determination of Fractal Dimension. All 300 x 210 px photographs of the chorioallantoic membrane were analyzed using the software ImageJ. The blood vessels on the image were first reinforced by being manually overlaid with black using any image - editing software to have a strong contrast against the background. These reinforced images were then converted to 8 - bit grayscale in ImageJ and the range of dark colors that represent the blood vessels were isolated from the background. The blood vessels were skeletonized and the fractal dimensions were retrieved using the box - counting method set to a maximum of 64 boxes on ImageJ.

Statistical Analysis. Kruskal-Wallis statistical analysis with a level of confidence of 0.05 was performed in order to compare the means of the average fractal dimensions of the treated eggs within and between treatments. If the asymptotic significance value is less than the desired level of significance of 0.05, it is declared that it is significant.

Results. – The means were calculated from fractal dimensions of each treatment of each replicate. A lower mean (See table 1) corresponds to a lower fractal dimension which suggests a stronger antiangiogenic effect, thus making the fractal dimension values inversely proportional to the antiangiogenic effect exhibited. The fractal dimension in anthocyanin-treated samples was lower compared to the quercetin-treated samples. This indicates that anthocyanin was more effective in inhibiting angiogenesis, only in terms of the values gathered, between the two substances being individual flavonoid treatments. The eggs that were not administered with any treatment displayed the highest fractal dimension mean of 1.306 among the five samples. Despite the consistency of values for the four samples, however, the calculated mean for dimethyl sulfoxide turned out to be higher than that of the samples treated with the combination of anthocyanin and quercetin. In theory, as the DMSO-treated is the positive control group, it must

Table 1: Fractal dimensions (Mean SD) of treatments after administration on chorioallantoic membranes of Darag native chicken embryos

Treatments	Mean SD	Description
No treatment	1.306 ±0.115	Control (-)
Quercetin	1.264 ±0.081	Weakest antiangiogenic effect
Anthocyanin	1.239 ±0.050	Moderate antiangiogenic effect
Anthocyanin + Quercetin	1.189 ±0.106	Strongest antiangiogenic effect
Dimethyl sulfoxide (DMSO)	1.240 ±0.053	Control (+)

exhibit the strongest antiangiogenic effect, but such was not the case. It also had a higher fractal dimension by 0.001 compared to the anthocyanin-treated experimental group which indicates that anthocyanin had a stronger antiangiogenic effect than DMSO, although the difference is negligible.

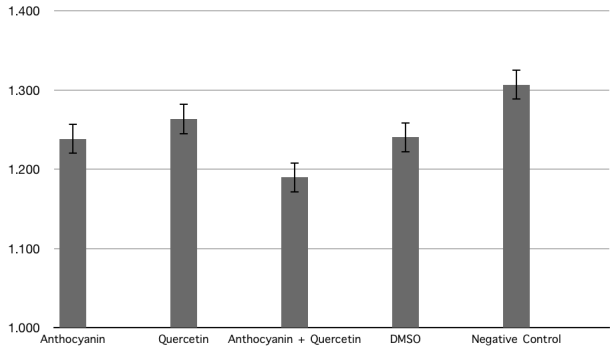

Fig. 1: Mean fractal dimension of each group

The Kruskal-Wallis statistical test was the primary test used for data analysis. Prior to the said test, the data set of replicate means for each treatment was initialized using the box-and-whisker plot by observing its distribution pattern and was found to be not normally distributed, which led to the choice of statistical test. For the data to be considered significant, the calculated asymptotic significance must be lesser than 0.05 and such was not the case as it turned out to be equal to 0.645 (See Table 2and 3).

In summary, between anthocyanin only, quercetin only, and their combination, the samples treated with combined anthocyanin and quercetin had the lowest blood vessel density as displayed by the low fractal dimension. Consequently, anthocyanin had a lower fractal dimension than quercetin, making it stronger as an antiangiogenic agent compared to quercetin in terms of numerical values. One irregularity in the data can be attributed to the fractal dimension of dimethyl sulfoxide being higher in value than that of the combination of anthocyanin and quercetin and almost equal to the dimension value of anthocyanin. Lastly, the data processed from the analysis was not considered significant.

Table 2: Kruskal - Wallis Test results from the fractal dimensional analysis of the skeletonized images of the samples

Treatment	N	Mean Rank
Treatment	N	Mean Rank
Quercetin	3	9.33
Anthocyanin + Quercetin	3	5
No treatment	3	10.33
DMSO	3	7.5
Total	15	

Discussion. – Flavonoids are recognized as chemopreventive agents to cancer and are the most abundant polyphenols in human diet. These molecules are especially known in inhibiting tumor growth by inducing apoptosis in multiple tumor cell lines and by halting cell-cycle [4, 12, 15, 19]. Multiple mechanisms have also been observed in which the flavonoids participate in that inhibit angiogenesis. In this study, the individual and combined performance of flavonoids anthocyanin and quercetin in inhibiting angiogenesis were assessed with the fractal dimension of blood vessels present on the CAM as parameter. The samples treated with flavonoids exhibited stronger anti-angiogenic effect than the negative control samples, but not to a statistically significant degree when the fractal dimension of the blood vessels were analyzed. In fact, no in-between group differences determined by this mode of assessment was statistically significant. Using the anti-angiogenic effect scoring method, however, significant in-between group differences were determined. For both methods, no synergistic effect was exhibited by the treatment with both anthocyanin and quercetin, but it is worth noting that the mean fractal dimension of all samples in this group is the greatest (when values from all replicates is averaged) based on fractal dimension means. This reveals that they manifested the mean fractal dimension of all samples in this group is the greatest (when values from all replicates is averaged) based on fractal dimension means. This reveals that they manifested the strongest anti-angiogenic effect, followed by the mean fractal dimension of samples treated with DMSO alone, albeit statisti-

Table 3: Test Statistics

Chi square	df	Asymp. Sig.
2.497	4	0.645

a: Kruskal Wallis Test
b: Grouping Variable: VAR0001

cally insignificant.

The possible mechanism behind the anti-angiogenic effect of the interaction between the two flavonoids is the antioxidant activity induced by both molecules in the chorioallantoic membrane. There have been reports that reactive oxygen species (ROS) stimulate the release of vascular endothelial growth factor (VEGF) signal protein [3] or mediate VEGF-induced signaling [1, 13, 25]. This is because ROS, specifically hydrogen peroxide (H2O2) which are produced by macrophages, enhance the expression of inducible nitric oxide synthase (iNOS), an enzyme responsible for the production of angiogenesis-mediating nitric oxide (NO) molecules (Han et al. 2001). Thus, antioxidants that scavenge H2O2 and superoxide down-regulate the expression of this enzyme and inhibit angiogenesis in vivo as a result (Polytarchou Papadimitriou 2004). Hidalgo et al. s (2010) study investigated the antioxidant activity of two interacting flavonoids by pairing all of the eleven (11) selected flavonoid groups, five of which are anthocyanins and two of which are quercetins. Each individual anthocyanin and quercetin has been found to have greater antioxidant capacity than when an anthocyanin is paired with a quercetin or vice versa . This indicates that the interaction has an antagonistic effect on the antioxidant capacities of the flavonoids. The suggested explanation for this phenomenon is that the hydrogen-bonding between flavonoids may have occurred, and reduced the availability of hydroxyl groups (-OH) in effect. Since the presence of hydroxyl groups is responsible for the neutralization of ROS, the antioxidant activity is reduced as a result of the interaction. The assay used in Hidalgo et al. s study, however, is the DPPH method which utilizes a radical distinct from hydrogen peroxide which is the one responsible for the stimulation of VEGF. Anthocyanin and Quercetin have both been shown to be able to scavenge H2O2 molecules, [26] making it possible that although hydrogen bonding may occur between the flavonoids, not the availability all hydroxyl groups is compromised, thus making the mixture still capable of neutralizing H2O2 molecules and inhibiting angiogenesis in the chorioallantoic membrane.

Following the strength of the observed anti-angiogenic effect of the mixture of anthocyanin and quercetin is that of DMSO had been confirmed to have anti-angiogenic effect on human aortic endothelial cells (HAECs) by decreasing the expression and activity of matrix metalloproteinase-2 (MMP-2) [10]. MMP-2 is an enzyme that breaks down the endothelial basement membrane to facilitate the growth of new capillaries. The down-regulation of these enzymes by DMSO had been found to be dose-dependent, causing significant anti-angiogenic effects at 2% and 3% DMSO concentration in the cell media DMEM-F12.

Conclusion. – The results of this study are not statistically significant in between fractal dimension means. However, the trends suggest that the combination of anthocyanin and quercetin exhibited the highest antiangiogenic effect in comparison to all the other treatments. Based on analysis, anthocyanin is more effective than quercetin in inhibiting angiogenesis. The three treatments were effective in inhibiting blood vessel growth. However, the mechanism of the treatments may not directly be about completely stopping the growth of blood vessels when a tumor grows. It could be that the treatments can only help in reducing the number of capillary branches or slowing down the rate of angiogenesis. The use of these treatments against angiogenesis can also be an indicator that foods that contain anthocyanin, quercetin, or the combination of both can also help in inhibiting angiogenesis for people suffering from angiogenesis-related diseases such as cancer and rheumatoid arthritis. In conclusion, the combination of anthocyanin and quercetin can control the growth and help in the process of inhibiting angiogenesis.

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