EFFECTS OF CRUDE EXTRACTS OF VARIOUS PIGMENTS ISOLATED FROM *Spirulina platensis* AND *Chlorella vulgaris* ON THE ANGIOGENESIS OF CHICK EMBRYO CHORIOALLANTOIC MEMBRANE (CAM)

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KEYWORDS

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ABSTRACT

This study was conducted to determine the angiogenic effects of different concentrations of phytopigments i.e. phycocyanin from *Spirulina platensis* and chlorophyll from *Chlorella vulgaris* on the chorioallantoic membrane (CAM) of developing chick embryo. Findings of study revealed that different concentrations of both crude phytopigments inhibit angiogenesis as shown by the continuous decrease of the vascular density index on the CAM of 10-day old chick embryo. However, Scheffe method showed that only 200 ppm and 300 ppm of crude phycocyanin and all experimental treatments of crude chlorophyll, including the 150 ppm, were able to significantly inhibit angiogenesis. It was also established that there is no significant difference in the angiogenic effects between the two phytopigments.

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1 Introduction

Angiogenesis is a process of new blood vessels formation and it controlled by certain chemicals produced in the body. Chemicals such as aspartame and serotonin stimulate cells repair and are also responsible for repairing of damaged blood vessels or formation of new ones (Surve et al., 2015; Yesildal et al., 2015; Banskota et al., 2016). Further, some chemicals such as heparin and endostatin are responsible for stopping the process of cell repairing and are also responsible for the inhibition of the synthesis of stimulatory chemicals, these inhibitory chemicals are known as angiogenesis inhibitors (Zhao-Na et al., 2015; Jin-Yan et al., 2016). Uncontrolled angiogenesis is a major contributor in a number of diseases such as arthritis, diabetes-related blindness, psoriasis, tumor growth and metastasis (Heidenreich et al., 2009; Gayetsky et al., 2014). Anti-angiogenic compounds would be useful in treating diseases caused by uncontrolled angiogenesis. Pro-angiogenic compounds are also required in wound healing and may be useful in minimizing tissue damage following ischemia damage from heart attacks or stroke (Mousa & Mohamed, 2004; Baharara et al., 2014).

Scientists and researchers are trying to use gene therapy to simulate this natural process by delivering different substances that may have potential positive or negative effects to angiogenesis of affected areas (Baharara et al., 2014). Various studies have been conducted to explore the possibilities of alternate anti-angiogenic substance based on herbal sources. Microalgae, such as *Spirulina* and *Chlorella*, received more attention from medical scientists as a nutraceutical and source of potential pharmaceuticals (Bishop & Zubeck, 2012; AbdEl Baky & El-Baroty, 2013). There are several studies which suggesting the ability of microalgae to inhibit viral replication, strengthen both the cellular and humoral arms of the immune system and cause regression and inhibition of cancers (Jensen et al., 2001; Tokusoglu & üUnal, 2003). *Spirulina* is a planktonic blue-green alga found in warm water alkaline volcanic lakes. It has a dark blue-green color, because it is rich in a brilliant blue polypeptide called phycocyanin. Hayashi et al. (2006) reported that phycocyanin affects the stem cells found in bone marrow. Medical scientists reported that *Spirulina* not only stimulates the immune system but also enhances the body's ability to generate new blood cells (El-kott et al., 2007; Kedik et al., 2011). On the other hand, *Chlorella* is a single-celled green alga, which has highest known levels of chlorophyll of any plant. Hyo-Jin et al. (2006) reported that the extract of algae has stimulatory effect on the physical stamina and acts as liver detoxifier, bowel cleanser and as catalyst for the absorption of essential elements (Hyo-Jin et al., 2006). *Chlorella* is also considered as one of the most powerful nutraceuticals (Kyadari et al., 2013). Therefore, present study has been conducted to find out the angiogenic potentials of the extract of microalgae *S.platensis* and *C. vulgaris* on the chorioallantoic membrane (CAM) of developing chick embryo.

2. Materials and methods

2.1 Collection and incubation of test embryos

A total of one hundred fifty two-day old fertilized hen's eggs were obtained from a poultry farm. The eggs were transferred to the Biology Research Laboratory of De La Salle University-Dasmariñas, Cavite and incubated at 37°C for another eight days.

2.2 Preparation of Algal extraction and determination of dry weight

Fresh *S. platensis* and *C. vulgaris* culture were obtained from the Aquaculture Department of the Southeast Asian Fisheries Development Centre (SEAFDEC) in Binangonan Freshwater Station located in the municipality of Binangonan, province of Rizal. The cultures were placed in an ice chest and transferred to the Biology Research Laboratory of De La Salle University-Dasmariñas, Cavite for the extraction procedure. Each microalgal culture was centrifuged at $4000 \times g$ for 5 min, pelletized, washed and resuspended twice with tap water. After washing, the supernatant was discarded and 40 mL of distilled water was added to the pelletized microalgae. The resuspended algal cells were then placed in a 50°C water bath for 24 h for autolysis. The autolysed cells were centrifuged at $10,000 \times g$ for 10 min and the supernatant was collected and dried in a rotary evaporator (Kightlinger et al., 2014). Two grams for each microalgae were used to determine their dry weight by using the formula (Boussiba & Richmond, 1979):

$$\% \text{ dry weight} = [(\text{pan weight} + \text{dried powder}) - \text{pan weight}] / \text{pan weight (not dried)}$$

2.3 Phytocyanin Assay

2.3.1 Phycocyanin assay

Using 100 mM phosphate buffer, 40 mg of extracted *Spirulina* was used for quantitative analysis of crude phycocyanin through a spectrophotometer at 620 nm absorbency level using the formula (Boussiba & Richmond, 1979):

$$\% \text{ crude CPC} = [A_{620} \times (10) \times (100)] / 3.39 \times (\text{mg sample}) \times (\% \text{ dry weight})$$

Where: 3.39 is extinction coefficient of CPC at 620 nm, 10 is total volume and 100 represent 100%.

2.3.2 Chlorophyll assay

Isolated chlorophyll level was quantified using 85% acetone and 50 mg of *Chlorella* extracted by spectrophotometer at 666 nm and 642 nm against an 85% acetone/water blank absorbency level using the formula (Boussiba & Richmond, 1979)

% crude CCA = $[A_{666} \times (10) \times (100)] / 9.93 \times (\text{mg sample}) \times (\% \text{ dry weight})$

Where: 9.93 is extinction coefficient of CCA at 666 nm, 10 is total volume and 100 represent 100%.

2.4 Preparation of different concentrations of crude phytopigments

Three different concentrations viz 150 ppm, 200 ppm and 300 ppm of *S. platensis* phycocyanin and *C. vulgaris* chlorophyll were prepared by double distilled sterile water. The desired concentrations were prepared from the crude extract obtained in the phytopigment assay using the dilution formula, $C_1V_1 = C_2V_2$, where C_1V_1 are the initial concentration and volume of crude phytopigment and C_2V_2 are the desired concentration and the final volume of crude phytopigment. The difference between the initial and final volume is the dilution factor used to obtain the desired concentrations.

2.5 Preparation of eggs for in situ sample application (West et al., 2000)

On the 4th day of incubation, the eggs were removed from the incubator and were placed in a 37°C oven to prepare them in situ sample application. Each egg was swabbed with 70% alcohol and using a 2-ml syringe, a small hole at the pointed end of the shell was made to puncture the air sac. Using a drill bit, a 0.5-cm square window in the shell was scored. The square shell was removed with a sharp pointed forceps to expose the CAM. The small square opening was covered by adhesive tape and each test egg was returned to the incubator horizontally, until treatment application on day 10.

2.6 In situ sample application (West et al., 2000)

At the end of the 10-day incubation, 72 viable eggs were selected, 36 eggs for each phytopigment, to be the test embryos, 9 for each treatments. In a laminar flow cabinet, through the small square opening of each test egg, 0.3 ml of the different concentrations of phytopigments and distilled water for the control group were administered. The opening was resealed and the eggs were returned to the incubator for further two days.

2.7 Data Collection and Statistical Analysis

Two days after the administration of crude phycocyanin and chlorophyll to the developing chick embryo, each of the test embryos was sacrificed and their CAM was prepared for observation of angiogenesis activity. The CAM was spread thinly in a petri dish and observed under stereomicroscope (Nikon®C-LED). Formed branch points or collaterals from four randomly selected areas of each CAM were counted and tabulated to determine the angiogenic effects of different concentrations of phycocyanin from *S. platensis* and chlorophyll from *C. vulgaris*.

To determine the significant difference in the angiogenic effects of different concentrations of crude phycocyanin from *S. platensis* and crude chlorophyll from *C. vulgaris* on the CAM of 10-day old chick embryos, one-way analysis of variance (ANOVA) was employed. Whenever there is significant difference, Scheffe method was used to compare individual treatment means.

3. Results and Discussion

Results of study revealed that the crude extract of phytopigments from *C. vulgaris* and *S. platensis* caused reduction in the formation and growth of blood vessels (Table 1).

Averages of 50.07 to 53.56 branch points were formed in the CAM of the tested embryos in the control treatment. Embryos treated with 150 ppm of crude phycocyanin extracted of *S. platensis* formed an average of 51.19 branch points while those treated with 200 ppm and 300 ppm of the crude extract formed an average of 26.13 and 21.27 branch points respectively, a decrease of more than half compared to that of the control treatment. Similar results were observed for the embryos treated with crude chlorophyll extracted of *C. vulgaris*. An average of 45.04 branch points were formed from the CAM of test embryos treated with 150 ppm of crude chlorophyll. While only an average of 25.54 and 20.54 branch points were formed for those treated with 200 ppm and 300 ppm of the crude extract, respectively.

Table 1 Average Number of Formed Branch Points Under the influence of various crude extracts .

Treatments	Average number of branch points	
	Applied with phycocyanin	Applied with chlorophyll
Control	53.56 ^A	50.07 ^A
150 ppm	51.19 ^{AX}	45.04 ^{BY}
200 ppm	26.13 ^{BX}	25.54 ^{CX}
300 ppm	21.27 ^{BX}	20.54 ^{CX}

Letters A and B compared the different concentration of each phytopigment while letter X and Y compared the two phytopigments per treatment. Different letters indicate significant difference at 0.05 level

Treated with different concentrations of crude phycocyanin

Treated with different concentrations of crude chlorophyll

Plate 1 Photomicrographs (Nikon® C-LED 45x) of the CAM of representative embryos treated with different concentrations of crude phytopigments: (A) control, (B) 150 ppm, (C) 200 ppm, and (D) 300 ppm.

Continuous reduction in the number of branch points formed was reported with increasing the concentration of the crude phytopigments, clearly indicated that both crude extracts had angiogenesis inhibition properties and signal the process to stop. Figure 1 shows the vascular density index of each treatment as they are compared with the control treatment.

Embryos treated with 150 ppm of crude phycocyanin had a percent negative of 95.58 decreased in the angiogenesis or a mean difference of -2.37 as compared to the test embryos of the control. While percent negative of 48.79 and 39.71 were the decrease in the angiogenesis for the test embryos treated with 200 ppm and 300 ppm or a mean difference of -27.43 and -32.29, respectively.

Figure 1 Vascular density index of test embryos treated with crude phytopigments as compared with the control.

Embryos treated with 150 ppm of crude chlorophyll had a percent negative of 90.67 decreased in the angiogenesis or a mean difference of -4.67 as compared to the test embryos of the control. While percent negative of 51.01 and 41.02 were the decrease in the angiogenesis for the test embryos treated with 200 ppm and 300 ppm or a mean difference of -24.53 and -29.53, respectively.

These results supported the claims that both phytopigments of *Spirulina* and *Chlorella* are effective in preventing diseases, such as cancer, brought about by uncontrolled angiogenesis (Tokusoglu & üUnal, 2003; Hyo-Jin et al., 2006; Kedik et al., 2011). Previous researches revealed that high concentrations of phycocyanin and chlorophyll inhibited the in vitro growth of tumor cell lines, which indicating that some tumor cell lines are directly sensitive to phycocyanin or chlorophyll. Further, it was reported that these cell lines were inhibited by inhibiting angiogenesis (Jensen et al., 2001; Kyadari et al., 2013; Saini & Sanyal, 2014). The number of branch points formed per treatment was also compared to determine the significant difference between crude extracts of phycocyanin and

chlorophyll as angiogenesis inhibitors on the CAM of 1chick embryos. Figure 2 shows which of the two phytopigments inhibit angiogenesis more effectively.

All three concentrations of chlorophyll induced greater inhibition to the angiogenesis on the CAM of the test embryos compared to phycocyanin. Embryos treated with 150 ppm crude phycocyanin formed an average of 51.19 compared to only 45.04 branch points formed in the test embryos treated with crude chlorophyll at the same concentration. Similar types of trend were observed with the other two concentrations viz 200ppm and 300ppm. Embryo treated with 200& 300 ppm phycocyanin showed an average of 26.13 & 21.27 branch points whereas only 25.54 & 20.54 for crude chlorophyll. However, using one-way analysis of variance, there was no significant difference between the crude extract of 200 and 300 ppm of phycocyanin from *S. platensis* and the extract of chlorophyll from *C. vulgaris* in inhibiting angiogenesis to CAM of the test embryos. This only shows that both phytopigments were effective angiogenesis inhibitors.

Figure 2 Average number of branch points formed in the CAM of test embryos from different experimental treatments.

Conclusions

Crude extract of phycocyanin and chlorophyll from *S. platensis* and *C. vulgaris* respectively, are angiogenesis inhibitors as shown by the continuous decrease of the vascular density index of the CAM of 10-day old chick embryo as their concentrations increased. Scheffe method reveals that 150 ppm of crude phycocyanin did not significantly inhibit the angiogenesis on CAM of chick embryo while 200 ppm and 300 ppm inhibit angiogenesis. On the other hand, using the same statistical method, all experimental treatments of crude chlorophyll were able to significantly inhibit the angiogenesis on the CAM of chick embryos. There is no significant difference in the angiogenic effects between crude phycocyanin from *S. platensis* and crude chlorophyll from *C. vulgaris* on the CAM of 10-day chick embryo, meaning that both phytopigments induced the same effect as angiogenesis inhibitors.

Conflict Of Interest

The authors declare that there is no conflict of interests that could possibly arise.

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