

# Cytotoxicity and Antiproliferation of Phycocyanin from *Spirulina platensis* Extract on WiDr Colon Cancer Cell Line

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**Abstract.** Phycocyanin from *Spirulina platensis* extract has anticancer activity against various types of cancer cell cultures. However study about its effect on colon cancer cell lines, especially the WiDr, has not been reported before. This study aimed to reveal the anticancer activity of phycocyanin from *Spirulina platensis* extract on WiDr cells. The research was an in vitro experimental study, with the investigation on cytotoxicity also antiproliferation as the anticancer parameters. Both cytotoxicity and antiproliferation test was conducted through MTT assay to observe the visualization and inhibition of proliferation of different concentrations of phycocyanin in several incubation times on the WiDr colon cancer cell line. The obtained data were then processed statistically with the Two Way ANOVA test at a significance value of  $p < 0.05$  and followed with the Post Hoc test since there were significant differences. Based on the results, it could be postulated that phycocyanin extracted from freshwater *Spirulina platensis* was classified as non-toxic ( $IC_{50}$  of 855  $\mu\text{g/ml}$ ). Consequently, it is less potential to be used as the treatment for colon cancer. However, phycocyanin could inhibit the proliferation of the WiDr cell for approximately 47.4%, specifically at the concentration of 1710  $\mu\text{g/ml}$  for 72 hours. It could be concluded that freshwater phycocyanin is less effective as an anticancer substance. The benefit of this study is to provide the new scientific evidence of the contrary results of freshwater phycocyanin activity from *Spirulina platensis* as an anticancer agent of colon cancer.

**Key words:** anticancer; colon cancer; freshwater phycocyanin

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## INTRODUCTION

Cancer is the second-highest cause of death globally and causes more than 8.8 million human deaths in 2015, which is equivalent to 1 in 6 deaths that occur in the world (Dewi, et al., 2018). The development of cancer (carcinogenesis) is a multistep process in which a normal cell will transform into cells that express the malignant phenotype. The process is a complete breakdown or dysfunction of controlled stages such as cell differentiation, proliferation, and programmed cell death (apoptosis) as well as the involvement of regular feedback mechanisms, thus causing the cancer cells' rapid growth as well as their metastasis (Nazih & Bard, 2018). Colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of cancer-related deaths worldwide, also one of the earliest, most molecularly characterized solid tumors (Athauda, et al., 2018). The sequence of development of CRC is gradually from adenoma formation to carcinoma through the accumulation of genetic and epigenetic events (Athauda, et al., 2018).

Cancer treatment with plants and plant-based products is a revolutionary method, besides being simple, safer, environmentally friendly, fast, more selective following its function and could act specifically on tumor cells without affecting healthy cells, it also tends to be non-toxic if compared to conventional treatment methods (Iqbal et al., 2017). One potential phytochemical is derived from several marine sources, mainly algae, because it is known to be able to modulate some cellular mechanisms such as cellular cytotoxics, prevent tumor cell invasion, and increase apoptosis of cancer cells (El-hack et al., 2019). It has been proven in many studies that microalgae activity can fight cancers (breast, ovary, skin, lung, kidney, and stomach) (El-hack et al., 2019; Ying et al., 2016), inhibit cell proliferation and reduce the ability to form colonies in some cancer cells.

*Spirulina platensis* was classified in the genus *Arthrospira* (Oscillatoriales) because it meets the phylogenetic and cytological criteria. *Spirulina* is blue-green, multicellular, and filamentous microalgae that have a symbiotic relationship with bacteria to bind nitrogen from the air. *Spirulina* is photosynthetic and

autotrophic algae. The primary photosynthetic pigment, phycocyanin, also contains chlorophyll and carotenoids (Vo, et al., 2015). Phycocyanin isolated from *Spirulina platensis* can cause the release of cytochromes C from mitochondria and caspase-dependent induction of apoptosis in HeLa cells (Nazih & Bard, 2018; Yang et al., 2014; Dewi et al., 2018). Ethanolic extract of 70% *Spirulina* showed significant cytotoxicity in K562 and Kusumi-1 cell cultures. IC<sub>50</sub> values obtained using blue trypan solution were 4.64 mg / mL for K-562 and 3.68 mg / mL for Kusumi-1 cell lines. Aqueous extract from *Spirulina* also showed cytotoxicity using the blue trypan method, at a higher dose, where the IC<sub>50</sub> value obtained was 12.68 mg / mL for K-562 and 2.13 mg / mL for Kusumi-1 cell lines. IC<sub>50</sub> value of 70% ethanolic extract *Spirulina* with the MTT test method was 0.40 mg / mL for K-562 and 0.31 mg / mL for Kusumi-1 cell lines. While the IC<sub>50</sub> value with the MTT test method for *Spirulina* water extract was slightly higher at 15.77 mg / mL for K-562 and 9.44 mg / mL for Kusumi-1 cell lines (Yohana, et al., 2016). Other studies also provide evidence of the anti-cancer activity of commercial *Spirulina* products against lung cancer cells, especially those related to the chemopreventive properties of *Spirulina* (Czerwonka et al., 2018). The cytotoxic effects of *Spirulina* ethanolic extract are quite similar to the effects of the use of cyclophosphamide, a chemical compound used as an anticancer agent. The cytotoxicity obtained from *Spirulina* extracts against cancer cell lines may be due to the presence of phytopigments (carotenoids, chlorophyll, and phycocyanin), as well as the polysaccharides that are constituents in these extracts (Yohana, et al., 2016). The results of other studies suggest that the combination of phycocyanin and all-trans-retinoic acid (ATRA) might have an inhibitory effect on HeLa cell growth by inhibiting cell cycle growth, inducing cell apoptosis, and promoting complement-mediated cytotoxicity (Yang et al., 2014). Besides, phytochemical screening results also showed the presence of tannins, flavonoids, steroids, glycosides, saponins, and alkaloids in *Spirulina platensis* ethanolic extract (Fithriani, et al., 2015). Antioxidant activity (IC<sub>50</sub>), antioxidant capacity (FRAP test) and total phenolic content found in the ethanolic extract of *Spirulina* sp. with successive values are 518.94 ppm, 49.95 ± 2.02 (µmol Fe<sub>2</sub> + eq.g-1 DW) and 0.32 ± 0.0025 mg GAE g-1 DW (Fithriani, et al., 2015). Based on the results of these previous studies, it could be concluded that *Spirulina* has the potential to be one source that can be developed into an anticancer drug (Yohana et al., 2016).

Colon cancer, as cancer with a high incidence in Indonesia, requires appropriate treatment for its sufferers, especially therapies with little side effects and

made from a natural product. Therefore, it is necessary to develop therapies from herbal ingredients that have the potential to kill cancer cells at the *in vitro* level. Phycocyanin from *Spirulina platensis* extract has anticancer activity against various types of cancer cell cultures. However, the activity of antiproliferation as one of the anticancer components of phycocyanin in WiDr colon cancer cell cultures has not been reported before. Therefore, this study aimed to reveal the anticancer activity of phycocyanin from *Spirulina platensis* extract on WiDr cells. After all, the benefit of this study is to provide scientific information regarding the potency of phycocyanin from *Spirulina platensis* extract as an anticancer agent for colon cancer.

## METHODS

### Cell lines

This study used the population of WiDr (colon cancer cell line) cell cultures that were in the living and confluent state and attached to cell culture flask. WiDr cells obtained from the Laboratory of Parasitology, Faculty of Medicine, Gadjah Mada University, were isolated, then cultured in cell culture flask using a complete medium (RPMI 1640 + FBS + fungizone + penstrep) and incubated in a CO<sub>2</sub> incubator at 37°C and CO<sub>2</sub> concentration of 5 %.

### Phycocyanin Preparation

Phycocyanin extraction from *Spirulina platensis* was carried out according to the method of Rahmawati, Hidayatullah, & Suprayatmi (2017), with modification. Furthermore, Phycocyanin was identified using high-performance liquid chromatography (HPLC) according to the modified method by Izadi and Fazilati (2018).

*Spirulina platensis* was collected from aquaculture ponds in Sukoharjo, Jawa Tengah, and separated from the growth medium. A total of 10 grams of *Spirulina platensis* was dried, then macerated in double-distilled water for 1 hour in a water bath at 26°C with a ratio of 1L of double-distilled water for 10 grams of *Spirulina platensis*.

The results of maceration thereafter were dried using a freeze dryer for 24 hours at a temperature of -4°C until reaching a yield of 40%. After freezing, the frozen sample was thawed at room temperature for 15 minutes. Afterward, the sample was centrifuged for 30 minutes at 3000 rpm.

*Spirulina platensis* extract was then processed by HPLC using a C18 column (250 x 4.6 mm) with a mobile phase in the form of a mixture of methanol and ammonium acetate 3% (ratio 7: 3, v / v) at 25°C and longwave of 615 nm.

### Cytotoxicity of Phycocyanin

Cytotoxicity analysis was performed using the MTT assay method, according to Wulandari, Meiftasari, Fadliyah, & Jenie (2018). WiDr cells were seeded in 96-well plate at a density of  $1 \times 10^4$  cells / 100  $\mu$ L/ well and were incubated in a CO<sub>2</sub> incubator at 37°C and CO<sub>2</sub> concentration of 5% for 24 hours.

Cells were treated with increasing concentrations of phycocyanin (1.95; 3.91; 7.81; 15.63; 31.25; 62.5; 125; 250; 500; and 1000  $\mu$ g / mL) and doxorubicin of 100  $\mu$ g / mL. Following 24 hours of incubation, the medium of culture was removed, and cells were washed with PBS (Sigma).

Afterward, cells were added with 100  $\mu$ L MTT (Sigma) 5 mg/mL, diluted with culture medium in each well for 4 hours. At this stage, the condition of the cell was examined and documented using an inverted microscope, mainly to determine the formation of crystalline formazone. When formazone crystals had formed, the test was continued by adding 100  $\mu$ L of stopper solution (10% SDS dissolved in 0.1 N HCl) to each well. WiDr cell cultures in plates were then incubated in a dark place overnight.

Later on, the absorbance of each well was measured using the ELISA reader at 595 nm (Bio-Rad) (Widiyastuti, et al., 2019). The single absorbance data was converted into the percentage of cell viability and was used to determine the IC<sub>50</sub> value based on (AAT Bioquest, 2019). The number of replications for each different treatment was four times.

### Antiproliferation of Phycocyanin to WiDr Cell Line

Antiproliferation analysis was also performed using the MTT assay method following the cytotoxicity procedure. The concentration range of the test substance given was  $\frac{1}{2}$  IC<sub>50</sub>, IC<sub>50</sub>, and 2x IC<sub>50</sub>. Moreover, the antiproliferation test was conducted in three different incubation times of 24, 48, also 72 hours.

After incubation, the absorbance reading for each well was performed using an ELISA reader with a wavelength ( $\lambda$ ) of 595 nm, to determine the percentage of cell viability, besides the percentage of inhibition relative to control (Wulandari et al., 2018).

### Statistical Analysis

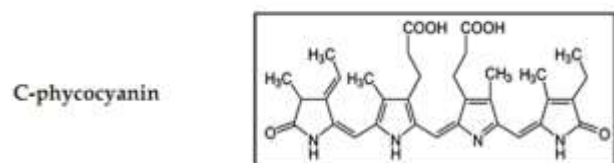
Data as the results of cytotoxicity and antiproliferation tests were processed statistically and presented in the form of images and figures as well as descriptive explanations. The absorbance value in the cytotoxicity test was analyzed using Microsoft Excel to calculate the percentage of cell viability, according to the following formula:

$$\% \text{ cell viability} = (\text{treatment absorbance} - \text{medium control absorbance}) / (\text{cell control absorbance} - \text{medium control absorbance}) \times 100$$

The cell viability values were tested in AAT Bioquest IC<sub>50</sub> calculator AAT Bioquest (2019), as followed Nikolova et al. (2019); Luparello et al. (2019), to obtain IC<sub>50</sub> levels, which then be used in antiproliferation testing. Cell viability calculations were also performed on the absorbance results in the antiproliferation test. The percentage of cell viability relative to control at incubation times of 24, 48, and 72 hours, was made to determine the inhibition among the treatments. The results of cell viability analysis in the antiproliferation test were continued with the Two Way ANOVA test at a significance value of  $p < 0.05$  and followed with the Post Hoc test since there were significant differences.

## RESULT AND DISCUSSION

WiDr cell was used as a signature type of colon cancer culture cell in this study due to its precise character, besides it has been developed and widely used in various anticancer activity studies *in vitro*. WiDr cells are cancer cells in humans that are isolated from colonic epithelial tissue in women aged 78 years. WiDr cells have p53 gene mutations and are responsive to chemotherapy (Ekowati et al., 2012). One of the characteristics is the overexpression of cyclooxygenase-2 (COX-2), where COX-2 expression can increase growth factor signaling, cancer cells proliferation, resistance to apoptosis, and metastases that lead to the development of colon cancer (Karpisheh et al., 2019; American Type Culture Collection, 2019; Ekowati et al., 2012). Phycocyanin-rich *Spirulina* is a reliable free radical scavenger that can inhibit lipid peroxidation in microsomes. Phycocyanin helps protect kidney failure caused by certain drugs, as well as having an anticancer activity (Udayan, et al., 2017).



**Figure 1.** Structure of Phycocyanin from *Spirulina platensis* (Ercolano, et al., 2019)

### Cytotoxicity of Phycocyanin from *Spirulina platensis* on WiDr Cell Line

According to the equation using the AAT Bioquest IC<sub>50</sub> calculator (AAT Bioquest, 2019), it was known that the IC<sub>50</sub> of phycocyanin on WiDr cell line was 855  $\mu$ g/ml (Figure 2.). The IC<sub>50</sub> value indicates the half-maximal inhibitory concentration that measures the effectiveness of a substance in inhibiting a specific biological or biochemical function. It represents the concentration of a substance or drug that is required

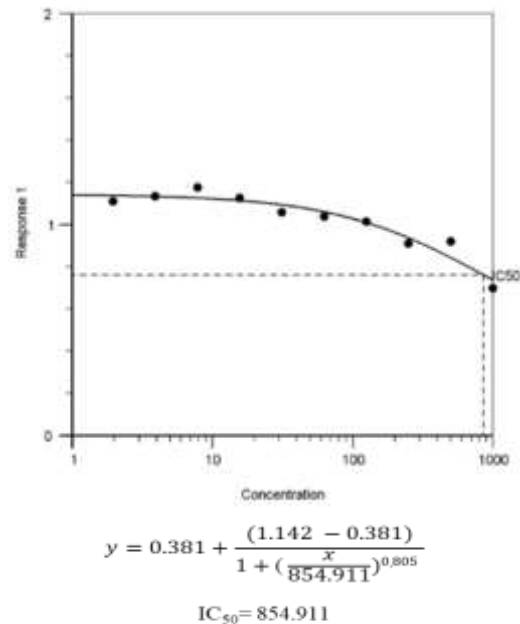
for 50% inhibition *in vitro* (Zaid, Hammad, & Sharaf, 2015). Therefore, the cytotoxic effect of phycocyanin is categorized as practically non-toxic (more than 500 µg/ml). However, this also means the substance of phycocyanin was not adequately potential for anti-cancer on WiDr cell, though this compound is safe to be ingested by the consumer.

The relatively non-potential effect of phycocyanin, possibly highly related to the source and habitat of *Spirulina platensis* that has been used in this study. The habitat and environment take large enrollments on the bioactive effect of the substances (Paliwal et al., 2017; Manirafasha et al., 2018; Papadaki et al., 2017). In this study, the *Spirulina platensis* had been cultivated on the freshwater population in Sukoharjo, Jawa Tengah, away from its origin. Microalgae, due to various environmental stresses, regularly adjust their cellular mechanisms to deal with them. The accumulation of the stress metabolites is closely related to the changes occurring in their metabolic pathways. The biosynthesis of metabolites can be triggered by some abiotic stresses like temperature, salinity, UV-radiation, and nutrient deprivation (Paliwal et al., 2017). The abiotic factor of pH, salt, and temperature would affect the microalgae to produce substances, which highly potential on some cancer cell line culture. Meanwhile, the freshwater cultivation of microalgae tends to produce fewer stress metabolites that effectively affect the cancer cell line culture. It is correlated with the study of Cahyaningrum (2019), which revealed that the extract of freshwater *Spirulina platensis* has the weak cytotoxicity of breast cancer culture cell of T47D.

### Antiproliferation of Phycocyanin

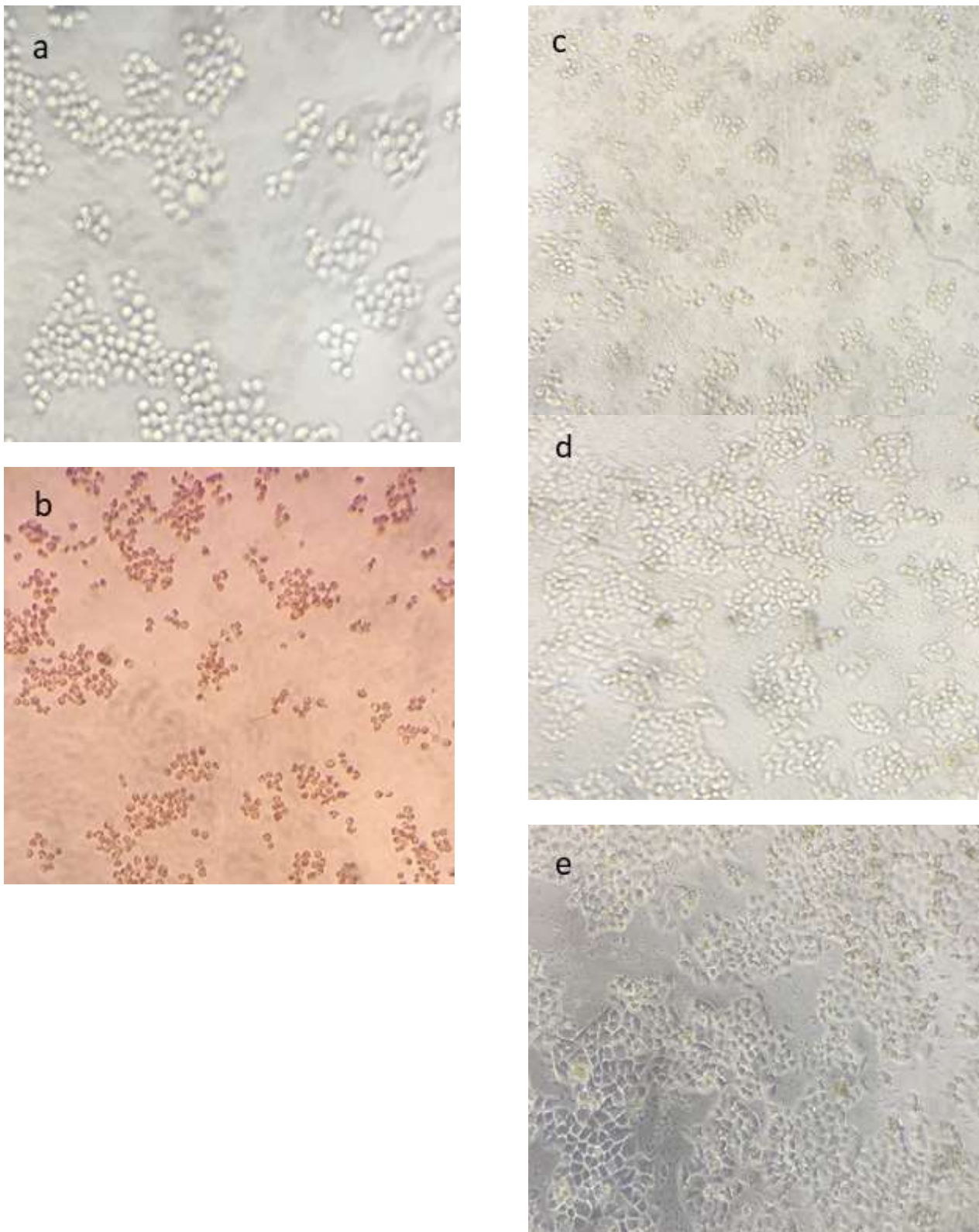
The antiproliferation activity of phycocyanin was conducted on various treatments of 1750 µg/ml, 855 µg/ml, and 427.5 µg/ml of phycocyanin, doxorubicin, control of cells, also medium of control. Based on Figure 3, it is known that amongst the treatments, the intervention of doxorubicin (100 µg/ml) showed most of the cell death. On the other hand, the treatments of 1710, 855, and 427.5 µg/ml phycocyanin indicated similarity in comparison with the control of the cell, which represents more living cells. Doxorubicin is recognized as a positive control due to its mechanism on various cancer cells. However, doxorubicin has several side effects, such as immunosuppression, nausea, and reversible arrhythmia. Moreover, long-term use of doxorubicin causes cardiomyopathy effect, followed by heart failure (Mutiah & Widyawaryanti, 2017; Wulandari et al., 2018). As shown in Figure 3, the dead cells were shrunk and compact; meanwhile, the live cells were rounded and clear on the center of the cell. The visualizations were related to the weak toxicity of phycocyanin; therefore, the

treatments of several concentrations of phycocyanin were not effectively destroying the WiDr cells. Moreover, the higher concentration of phycocyanin could increase the death of the cell. Nevertheless, it would visually be acclaimed that the control cell has a similar appearance in comparison with the cells that had been treated with the phycocyanin.

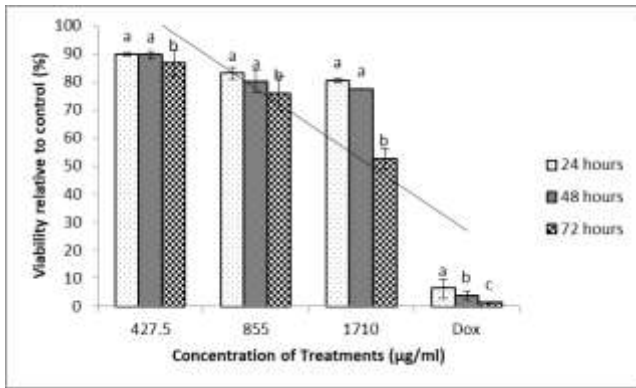


**Figure 2.** The IC<sub>50</sub> equation of phycocyanin on WiDr colon cancer cell line

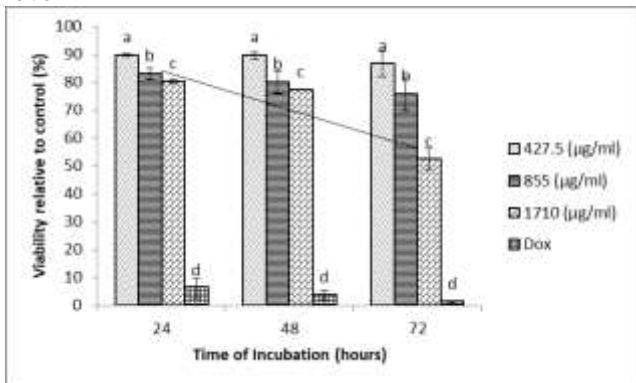
To examine the anti-proliferation activity of phycocyanin with the consideration of dose- and time-dependent incubation; therefore, the percentage of cell viability relative to control amongst the treatments was analyzed as presented in Figure 4. Based on Figure 4a., it is shown that the longer the time of incubation would decrease cell viability up to 52.6% at the concentration of 1710 µg/ml after 72 hours; hereafter, the incubation significantly ( $P < 0.05$ ) affects the viability of the cell. Though the results of 24 and 48 hours of incubation were not significantly different, the incubation time of 72 hours was significantly the most effective time for treating the WiDr cell with phycocyanin. The results also represent the effect of phycocyanin to reduce the cell cycle at the concentration above 1000 µg/ml, as stated by Czerwonka et al. (2018). It is clearly described in Figure 4b., the higher the concentration of phycocyanin treatments, could decrease the cell viability significantly ( $P < 0.05$ ). Among the various concentrations, the treatment of two times IC<sub>50</sub> of 1710 µg/ml was the most potent to lessen the viability of the WiDr cell. However, the treatment of phycocyanin could not compete for the efficiency of doxorubicin, which has decreased the cell up to 1.3%. It is in line with the non-toxic character of freshwater phycocyanin used in this study.



**Figure 3.** Visualization of WiDr cells in several treatments (a) control cell; (b) doxorubicin of 100 µg/ml; (c) phycocyanin of 1710 µg/ml; (d) phycocyanin of 855 µg/ml; and (e) phycocyanin of 427.5 µg/ml.



(a) Different font indicates significance at the 0.05 level



(b) Different font indicates significance at the 0.05 level

**Figure 4.** WiDr Cell viability in (a) treatments of doxorubicin and phycocyanin of 1710, 855, and 427.5 µg/ml; (b) various incubation times of 24, 48, and 72 hours.

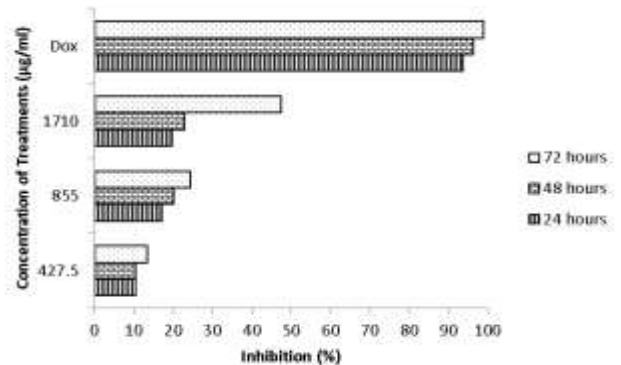
The proliferation of cancer cells is determined and controlled by the mechanism of the multiplying process of cell division. Cell proliferation phases M, G1, S, G2, are regulated by genes, protein stimulators, and proliferation inhibitors (CDK protein groups). So, if there is damage to the regulation of cell proliferation, it would form uncontrolled cell growth or cancer. In general, the mechanism of antiproliferation is adjusted to the phase of cell division, where p53 protein is responsible for stopping the cell cycle in the G1 phase while the S phase could be cut cycles through induction replication stress (RS) (Thangam et al., 2013). Besides, the inhibition of the formation of spindle yarn might be used as a barrier to the cell cycle in the G2 phase (Thangam et al., 2013).

**Percentage of Phycocyanin Inhibition**

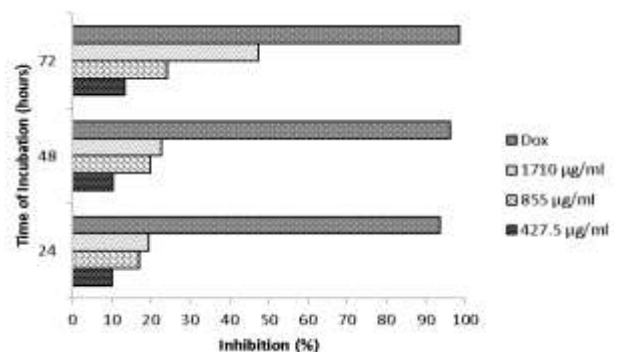
The percentage of inhibition activity of phycocyanin to WiDr cell is presented in Figure 5. The results showed that the most effective inhibition (47.4%) was observed when cells were treated with the highest phycocyanin concentration (1710 µg/ml) after 72 hours of incubation. However, these results could not be compared with the doxorubicin treatment that inhibited WiDr cell up to 98% after 72 hours of incuba-

tion. This result is in line with previous studies that stated that the longer time of incubation also the higher the dose of phycocyanin would increase the inhibition of culture cancel cell (Thangam et al., 2013; Ravi et al., 2015; Yang et al., 2014).

The results of this study showed that the treatment of phycocyanin has weak antiproliferative activity on WiDr cells. It is reasonably due to the use of freshwater phycocyanin obtained from the cultivation of *Spirulina platensis*. The content of chlorophyll and phycocyanin mainly influences the color of *Spirulina platensis* biomass. Freshwater *Spirulina platensis* could produce phycocyanin with content and morphology that are almost similar to marine phycocyanin (Ekantari, Marsono, Pranoto, & Harmayani, 2017). *Spirulina platensis* from freshwater aquaculture consists of phycobiliprotein pigment-containing phycocyanin pigment with a maximum λ value of 615 nm. Based on the absorption at the maximum wavelength obtained by measuring the absorption of the solution at a wavelength of 650 nm, it could be calculated levels of phycocyanin pigments, which is equal to 6.6663 mg / g wet *Spirulina platensis* weight biomass (Agustini, 2007).



(a)



(b)

**Figure 5.** The inhibition activity of phycocyanin on WiDr cell in (a) vary treatments of doxorubicin, phycocyanin of 1710 µg/ml; 855 µg/ml; and 427.5 µg/ml; (b) several incubation times of proliferation of 24, 48, and 48 hours.

Cultivation is one of the effective methods to fulfill the demand for natural products. However, it is often a lack of targeted metabolites that lead to less effectiveness. As expressed in this study, the freshwater phycocyanin acted less effectual rather than marine phycocyanin as an anti-cancer agent (Czerwonka et al., 2018; Konícková et al., 2014; Zaid et al., 2015; Yohana et al., 2016).

Although many pieces of evidence through several studies postulated positive anti-cancer activity of phycocyanin, in this research, exceptional circumstances limited the multifunctional usage of phycocyanin, particularly to overcome colon cancer treatment. Thereafter the information could provide the new evidence of the contrary results of freshwater phycocyanin activity from *Spirulina platensis* as a chemopreventive agent. The results would be useful to develop an advanced technique to produce freshwater phycocyanin with anti-cancer activity as its wild type.

### CONCLUSION

In conclusion, phycocyanin extracted from freshwater *Spirulina platensis* was classified as non-toxic (IC<sub>50</sub> of 855 µg/ml). Consequently, it is less potential to be used as the treatment for colon cancer. However, phycocyanin slightly promotes anticancer activity by inhibiting the proliferation of the WiDr cell for approximately 47.4%, significantly at the concentration of 1710 µg/ml for 72 hours. It can be concluded that freshwater phycocyanin is less effective as an anticancer substance of colon cancer.

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