

PURIFICATION OF ANTIANGIOGENESIS COMPOUND FROM INDONESIAN SHARK CARTILAGE (*Elasmobranchii*) AND *IN VITRO* ASSAY WITH ENDOTHELIAL CELL CULTURE*

Dondin Sajuthi^{1,2,3,4}

²Department of Chemistry, Faculty of Mathematics and Sciences of Bogor Agricultural University

³Primate Research Center of Bogor Agricultural University

⁴Biopharmaca Research Center of Bogor Agricultural University

ABSTRACT

Shark cartilage has been recognized as an alternative medicine for cancer and other diseases caused by the angiogenesis process. In this study, the antiangiogenesis effect of crude extract, purified glycoprotein and polysaccharide of shark cartilage were analyzed at various concentrations (400, 600 and 800 ppm). Antiangiogenesis assay was measured by analyzing the proliferation inhibition of capillary pulmonary artery endothelium cell culture (CPAE-ATCC CCL-209).

Chromatography gel filtration with Sephadex G-75 was used to extract the glycoprotein compound. The process yielded 17.4% as crude glycoprotein. Further purification was performed with DEAE Sephadex A-25. Fractionation based on ultraviolet absorption at 280 nm and qualitative assay resulted into four fraction and yielded 0.009% - 0.32% of fraction. Extraction and fractionation of polysaccharide were performed by gel filtration with Toyopearl HW-65F. Three fractions were collected and yielded 0.61% - 2.26% of fraction.

The result of *in vitro* antiangiogenesis assay showed that crude extract and fractions of glycoprotein and polysaccharide gave an inhibition on the proliferation of endothelial cell culture. The crude extract of glycoprotein (800 ppm) strongly showed 100% proliferation inhibition. From the polysaccharide fraction, fraction III that contain the highest concentration of polysaccharide and the lowest concentration of protein showed 98.9% - 100% of inhibition. This study indicates that purified compound from Indonesian shark cartilage has potential *in vitro* antiangiogenesis effect.

Key Words: Shark Cartilage, Antiangiogenesis, Endothelial Cell

INTRODUCTION

Indonesia's maritime is a rich source for biopharmaca product. Shark as one of the popular fish from a large number of species in the ocean believed to have potential as an anticancer compound. It has been used as a remedy in a conventional form or in modern packaging. The use of Indonesian shark cartilage as an alternative medicine is not common. Shark is captured more for their fins for a very popular delicacy soup "shark fin soup". Their meat and bones often goes to waste.

Information on the purification and bioactive compound with specific antiangiogenesis activity will increase the economic value of shark.

It is known that the skeleton of shark is made of pure cartilage. The cartilage is formed from protein and complex carbohydrates and strengthened by certain fibers without supply of nerve and blood vessel (Lane, *et al.*, 1992). Angiogenesis, the development of a new blood supply, plays an important role in the process of embryo's growth, healing, tumoral growth and metastases dissemination (Folkman, *et al.*, 1992). In abnormal

¹To whom correspondence should be addressed, phone 62-251-313637

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condition, angiogenesis can dominate and support establishment of serious diseases such as cancer, rheumatoid arthritis, diabetic retinopathy (Maugh, 1981), psoriasis (Dupont, *et al.*, 1998), and liver cirrhosis (Norrby, 1997, Jackson, *et al.*, 1997). Physiologically, the angiogenesis process rarely occurs in a normal adult except in ovarium, endometrium and placenta (Norrby, 1997). Kerbel (1997) indicated that the development of new vessels stimulates by an angiogenesis stimulator and also the inhibition on the production of an angiogenesis inhibitor. Therefore, antiangiogenesis strategy can be used in developing an anticancer agent (Ono, *et al.*, 1996) and other diseases dominated by angiogenesis (Norrby, 1997).

Evidence of shark cartilage has antiangiogenesis activity were reported by Lee and Langer (1983) by inhibiting the vascularization of rabbit's cornea implanted with V2-carcinoma cells. Crude extracts from Indonesian shark cartilage of subclass Elasmobranchii, species *Alupias pelagicus* (Lost & Stevens, 1994) has been proofed to have the capacity in inhibiting the vascularization of chicken embryonic chorioallantoic membrane (Sajuthi *et al.*, 1999).

The objective of this study is to obtain purified bioactive compound from Indonesian shark cartilage. For better understanding on the antiangiogenesis mechanism, the activity of the purified compound will be measured *in vitro* by evaluation of proliferation inhibition of capillary endothelial cell culture. The initiation of neovascularization starts with the forming of endothelial cell. Therefore, the use of endothelial cell is to evaluate whether the antiangiogenesis effect through the inhibition of the cells is happening.

EXPERIMENTAL METHODS

Materials

Materials were obtained from the following sources: Indonesian shark cartilage of species *Alupias pelagicus* from South of West Java made into powder, DEAE Sephadex A-25 and G-75, Toyopearl HW-65F, Fetal Bovine Serum (FBS, Hyclone), Dulbecco's Minimum Essential Media (DMEM, GIBCO), Lowry, Molisch, Bradford, and Ninhydrin reagent, Phosphat Buffered Saline (PBS, GIBCO) and Capillary Pulmonary Artery Endothelial Cell (CPAE) ATCC CCL-209 (Hay, *et al.*, 1988).

Extraction and Purification of Glycoprotein

The fractionation methods was developed using methods of Kwon, *et al.*, (1996) and Tsuno, *et al.*, (1994). Powdered shark cartilage was extracted by maceration technique with 100 mM Tris-HCl buffer pH 8.5 followed by agitation for 24 hours at 4°C. This mixture then centrifuged at 35.000 g for 20 minutes. Supernatants subjected to gel filtration on a column of Sephadex G-75. Fractions collected every 5 ml in a tube and monitored by UV absorption at 280 nm. Qualitative analysis of protein and polysaccharide measured with Bradford, Lowry, and Molisch reagent. Qualitative analysis for amino acid measured by Ninhydrin reagent. The absorbance value and qualitative assay result will be use for grouping of glycoprotein fraction. Fraction with high of protein content were collected, concentrated and freeze-dried as crude glycoprotein fractions at certain molecular weights. Further purification of crude glycoprotein performed with DEAE Sephadex A-25 and eluted with 10 mM of Tris-HCl buffer containing 0.1 M NaCl. Then eluates were subjected to dialysis with BM cut off 3,500 for 48 hours at 4°C. Freeze-dried dialysate will be used for *in vitro* assays.

Extraction and Purification of Polysaccharide

Extraction and fractionation of polysaccharide are based on the method of Wang, *et al.*, (1993). The powdered shark cartilage is dissolved in aquadest with ratio of 1:4. The mixture was heated at temperature of 60°C for 4 to 6 hours, filtered with cotton then centrifuged at 12,000 g for 10 minutes. The precipitate is extracted with ammonium oxalate and NaOH 5% until it forms a precipitate and filtrate. The filtrate is then subjected to dialysis process to water for 12 hours. The dialysate is purified by gel filtration with Toyopearl HW-65F and NaOH buffer as an eluent. Fractions are collected every 5 ml with the same method for glycoprotein. The highest polysaccharide and the lowest protein content were used as criteria for selection of fraction.

In vitro Assay CPAE Cell Culture

Using a six well plate, add 10^4 of endothelial cells in each well, the methods used for this assay is a modification of Moses, *et al.*, (1990). These cells cultured with DMEM supplemented with 20% FBS and antibiotic (penicillin-streptomycin 1%) incubated for two days or till 30% confluence. Shark cartilage extracts put in to each well with various

concentrations (400, 600, and 800 ppm). All fraction were performed in triplicate. Cells evaluated every day with inverted microscope. On day 5-7 (depends on the 100% confluence of cell control) the quantity of viable cells were counted with Neubauer counter for each samples.

RESULT AND DISCUSSION

Extraction and Fractionation of Compound

As the first step, crude glycoprotein was extracted with Tris HCl buffer pH 8.5, then precipitated by centrifuge at 35,000 g. Supernatant is subjected to chromatography on a gel filtration column with Sephadex G-75 to isolate molecules with molecular weight (MW) ranging from 1000 to 100,000. Protein was separated based on the value of absorption of protein concentration monitored by UV absorbance at 280 nm (Deucher, 1990). Three fractions of glycoprotein were obtained: Fraction A with the lowest absorption values ($A < 0.5$), Fraction B with the moderate absorption values ($0.5 < A < 1.2$) and fraction C with the highest value of absorption ($A > 1.2$). The relation between absorption and fractions

obtained from the gel filtration Sephadex G-75 shown in Figure 1.

Each fraction of A, B and C is divided in two then pooled as crude glycoprotein for *in vitro* assay of antiangiogenesis effect and for further purification. Further purification by ionic exchange chromatography column DEAE Sephadex A-25 as a negative ionic exchanger is used. Fractions applied to the column and eluted by buffer to determined proteins that were not being held in the column. Figure 2 shows result of fractionation of fraction C with the highest protein concentration.

Figure 2 shows that proteins with negative polarity are divided into three fractions. Fraction I with the lowest protein concentration ($A < 0.36$), fraction II has moderate protein concentration ($0.36 < A < 0.7$). The third fraction has the highest concentration of protein. Fractionated after tubes number 29 has the lowest concentration ($A < 0.05$) and put in fraction IV. All purified fractions (I, II, III and IV) performed for antiangiogenesis assay with endothelial cells.

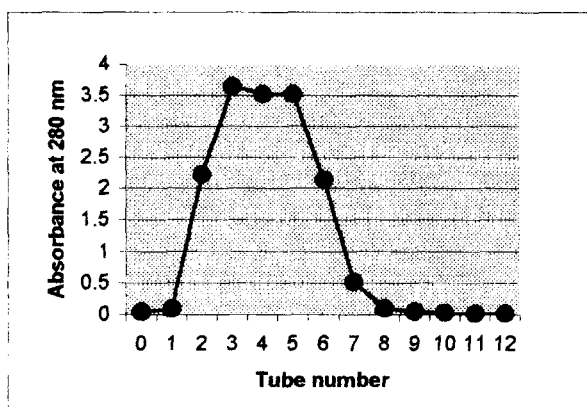


Figure 1. The relation of absorption and fractions obtained from the chromatography gel filtration Sephadex G-75.

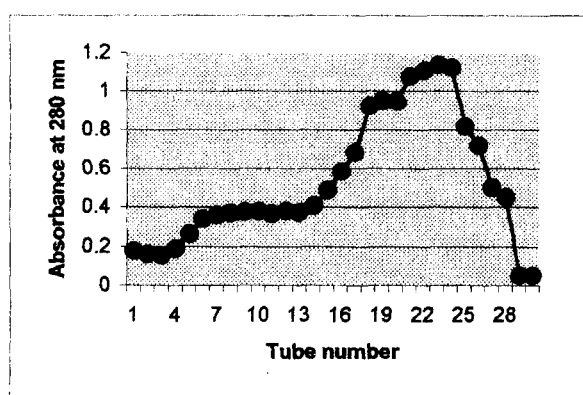


Figure 2. Glycoprotein purification of fraction C by DEAE A-25 ion exchange chromatography.

Table 1. Qualitative analysis of polysaccharide

Fraction	Reagent			
	Molisch	Barfoed	Bradford	Lowry
I	Low	Low	High	High
II	Moderate	Moderate	Moderate	Moderate
III	High	High	Moderate	Low

Crude extract glycoprotein yield was 1.29%-1.5%. Results of glycoprotein fractions were 0.27%-0.29%, 0.009-0.012%, 0.30-0.32%, and 0.005%-0.009%, respectively for fraction I, II, III and IV. For fraction III has a high concentration of protein and gave the highest yield. Therefore as an illustration, from 100 g of shark cartilage, we could have 0.30 g glycoprotein fraction and 1.50 g of crude glycoprotein.

There were two groups of polysaccharides compound resulted from this experiment e.g. soluble and non-soluble in ethanol. Sajuthi, *et al.*, (1999) found that non-soluble fraction showed higher antiangiogenesis activity than the soluble fractions. Non-soluble polysaccharide fractions divided into three groups based on the combination of protein and polysaccharide content using qualitative assay. Qualitative analysis for polysaccharide is Molisch and Barfoed reagent. Qualitative analysis of protein is Bradford and Lowry reagent. Fraction I has a low concentration of polysaccharide but high content of protein. Fraction II has moderate concentration of polysaccharide and protein. For fraction III, it has the high concentration of polysaccharide and low protein. This data is shown in Table 1.

Yield of polysaccharide fractions as follows 0.61-0.65%, 2.17-2.26% and 1.92-2.24%, respectively for fraction I, II and III.

***In vitro* Antiangiogenesis Assays on Endothelial Cell Culture**

In vitro assays performed on glycoprotein fractions were crude extract of glycoprotein, fraction I, II, III and IV. Fraction I, II, and III of polysaccharide were also assayed. All fractions were evaluated with the same concentration at 400 ppm, 600 ppm and 800 ppm. Selection of concentration based on preliminary study by Sajuthi, *et al.*, (1999). PBS and culture media were used as negative control. The activity of antiangiogenesis of the fractions evaluated was based on the capacity to inhibit the growth of endothelial cells on the treated samples compared to the control well. More than 50% means the fraction has capacity to inhibit the growth of cell toward death. Less than 50%, the fraction tends to only inhibit the growth of cells (slowing the rate of replication). The percentage of inhibition from each fraction can be seen in Figure 3.

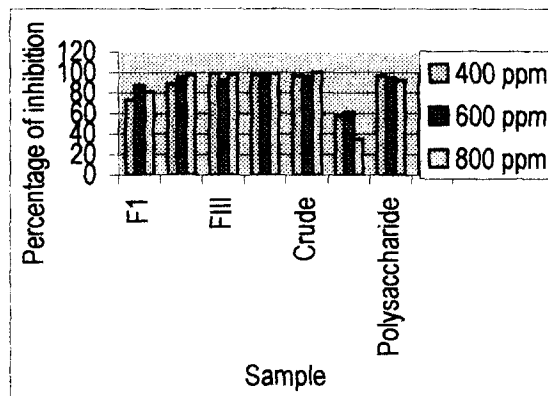


Figure 3. Antiangiogenesis activities for glycoprotein and polysaccharide fractions on CPAE-ATCC CL 209 cell culture.

This *in vitro* assay shows that almost all fractions with all concentrations (except fraction I of polysaccharide with concentration 800 ppm) gives strong inhibition on the cell proliferation. McGuire, *et al.*, (1996) proofed also that the antiproliferative effect of shark cartilage was specific for vascular endothelium. Crude glycoprotein extract had the highest growth inhibition of cell (100%) and the highest yield. Fraction IV of glycoprotein showed high inhibition but contain low protein and low yield.

Fraction I of polysaccharide showed the lowest inhibition even though it has the highest concentration of protein. This unexpected result might be due to the heating (over 60°C) during early extraction process.

CONCLUSION

This study showed that all fractions of glycoprotein and polysaccharide have *in vitro* activity to inhibit the growth endothelial cell culture. Therefore, it could be postulated that one of mechanism of antiangiogenesis effect of shark cartilage is through the inhibition of the endothelial cell growth. Further study on the type of cancer that can be treated by using the shark cartilage is needed.

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