ORIGINAL ARTICLE

Purification and Antioxidant Activity Assay from *Scorpaenopsis diabolus* Toxin

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ABSTRACT

Introduction: Long term consumption of antioxidant supplements has been shown to be related to adverse effects such as increased risk of death from synthetic antioxidant compounds used. Of all Indonesia's marine wealth, *Scorpaenopsis diabolus* is still rarely studied regarding its bioactive components in its venom. To evaluate the purification process and the potential of *S. diabolus* toxin as natural antioxidant. **Materials and Methods:** Toxins were extracted by batch method using phosphate buffered-saline and purified using FPLC system with anion exchanger column. In order to identify the concentration and molecular weight of protein, Lowry analysis and SDS-PAGE test were carried out. The protein was then assayed for toxic activity using the Brine Shrimp Lethality Test and assayed for antioxidant activity using DPPH method (2,2-diphenyl-1-picrylhydrazil). **Results:** Ten purified protein fractions were obtained from S. diabolus toxin with the highest concentration in 101.1 kDa fraction from 0% salt elution. The highest toxic activity was found in 25 – 225 kDa fraction with LC50 = 27.68 µg/mL, while the antioxidant activity varied from weak to strong with IC_{50} values ranging from 64 – 179 µg/mL, but with a negative inhibition percentage, except for protein with an estimated molecular weight of ~10 kDa at 50 ppm. **Conclusion:** Based on its IC_{50} value, *Scorpaenopsis diabolus* venom has the potential to be developed as antioxidants. However, research regarding the antioxidant activity of other protein fractions still need to be done, and further study using more advanced antioxidant testing method is required, as most of the inhibitory activity are negative.
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INTRODUCTION

In this era, techological advances bring major impact on human life, including lifestyle changes especially in urban areas. In recent years, there has been an increase in the number of factories and motorized vehicles usage. However, these rapid changes are having a serious effect on environment from the waste generated by factories and poisonous fumes from vehicles. Polluted environment has led to ozone depletion that causes increasement of free radicals' exposure from solar radiation. Aside from solar radiation, human body can also be exposed to free radicals through tobacco smoke and unhealty food. Data from several studies suggest that excessive exposure to free radicals leads to cell damage and oxidative stress that triggers chronic diseases, including cancer, cardiovascular disease, autoimmune disease, etc. In Indonesia, cardiovascular disease and cancer are the top two causes of death from disease in in 2016 with 35% of death caused by cardiovascular disease and 12% of death caused by cancer from the total of 863.000 deaths(1).

Previous research has established that human body naturally produces antioxidants that has the ability to

inhibit free radicals' activity and to counteract oxidative stress. To enhance the antioxidant effect, it is required to consume food rich in antioxidant and/or antioxidant supplement. For people with more intense activities, antioxidant supplements are more preferred due to its practicality. Yet, antioxidant supplement consumption in long term or in high dose can be harmful to human, with the potential to cause other diseases, including cancer and stroke(2), and interaction with other medicine taken that causes adverse effects.

Indonesia is an archipelagic country and the second biodiversity country in fisheries after Brazil with 8,000 species of fish, consisting of 1,300 freshwater fish species or 10% of the total freshwater species in the world(3). Yet, there are still many marine species that have not been explored further, including the poisonous fish species. *Scorpaenopsis diabolus* from the *Scorpaenidae* family is a type of scorpionfish that is widely distributed in Indo-Pacific ocean(4). There is a growing body of literature that recognises *Scorpaenopsis diabolus* toxin found in the dorsal and anal fins that might causes serious injuries(5). Scorpaenopsis diabolus also posses a characteristic in the form of bright colors on the inside of pectoral fins that is used to scare off predators. Nevertheless, researches on *Scorpaenopsis diabolus* is still limited compared to other species from the same family, including lionfish, stonefish, and other scorpionfish(6).

To date, a number of studies have investigated the antioxidant activity of *Scorpaenidae* species' venom, including *Pterois volitans*(7) of lionfish species and *Scorpaena notata*(8) of scorpionfish species. Meanwhile, the venom apparatus and pharmacology of poisonous fish are similar in most species, despite a wide taxonomic range(9). Therefore, it is suggested that there is a possibility of similar antioxidant activity on *Scorpaenopsis diabolus* species. This study aims to examine the antioxidant activity of *S. diabolus* protein venom as an alternative source of natural antioxidants to replace the role of antioxidant supplements with synthetic ingredients.

MATERIALS AND METHODS

Venom Extraction and DNA Verification

Scorpaenopsis diabolus obtained from Kepulauan Seribu, Indonesia, were chilled in approximately -20°C for 40 minutes. The pectoral fins were cut using sterile scissors, immersed in 70% ethanol, and the species was verified by DNA Barcoding method using CO1 gene. The DNA testing procedure was carried out by extracting genomic DNA using the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (FAVORGEN, Taiwan), DNA amplification using PCR method using MyTaq HS Red Mix-Bioline polymerase enzyme (Meridian Bioscience, USA), DNA barcoding with CO1 target gene, and DNA sequencing using single pass DNA sequencing.

Afterwards, venom extraction process were done using batch extraction method(10) by cutting the dorsal and anal spines using sterile knife, then immersing the spines in phosphate buffered-saline (PBS) solution (pH 7.4) at 4°C of twice the mass of the spines, stirred, and stored in 4°C refrigerator. Storage of spines was done for 1 week so that the toxin from the spines can be properly dissolved into the solution. PBS which now contains fish toxin components was then put into a microtube for the centrifugation process. Centrifugation was carried out at 4°C at a speed of 14,000 g to remove insoluble contaminants. Supernatant (crude venom) was then stored at -80°C, the optimal temperature to maintain the hemolytic activity of fish toxin(11), until further use.

Ethical Approval

The animals (*Scorpaenopsis diabolus* and brine shrimps; *Artemia salina*) used in this study were commercially sourced thus the owner's consent was not required.

Crude Venom Purification

To determine whether there are proteins contained in the venom, *S. diabolus* crude venom were purified by Fast Protein Liquid Chromatography method using 5 mL Strong Anion Exchange HiTrap Q HP column (Cytiva, USA). Since scorpionfishes' venom pH ranges from 7-8(12), buffer solutions at this pH range were used, namely 20 mM phosphate-buffer with 0.1 M NaCl at pH 7.5 as running buffer and the running buffer solution added with 1 M NaCl as elution buffer(10) . Protein purification was carried out using FPLC ÄKTA Start (Cytiva, USA) with UNICORN™ START software. Prior to purification process, the column needs to be washed with 70% ethanol to remove residual protein from previous use. Subsequently, the running buffer and elution buffer are run through the column to assure that impurities and residual ethanol have left the column. The concentrations of elution buffer were varied from 0%, 20%, 40%, 60%, 80%, and 100% to see the protein fraction that could be eluted for each salt concentration. Eluted protein fractions were then contained in microtubes and kept in freezer for further use.

Protein Concentration Estimation

Crude venom and protein fractions from purification process were assayed for its concentration using Lowry method with Bovine Serum Albumin (BSA) 50 – 350 µg/mL as standard and aquadest as blank. 10µL Lowry reagent made from 100 mL Na_2CO_3 in 0.1 N NaOH and 1 mL $CuSO_4$ 1% with 1 mL NaK-Tartrate 1% was added to 2 µL standard solution, blank, and samples. All solutions were incubated for 10 minutes at room temperature, then added 1 µL Folin-Ciocalteu reagent to each solution, followed by 30 minutes incubation. The absorbance of each solution was measured using Nanodrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) at 650 nM due to its limited amount of sample.

SDS-PAGE

Alongside Lowry analysis, crude venom and the purified protein fractions from S. diabolus venom were assayed by SDS-PAGE (Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis) in order to identify its molecular weights. SDS-PAGE test was carried out using 5% stacking gel and 12% separating gel with PageRuler Prestained Protein Ladder marker (Thermo Fisher Scientific, USA) that is able to detect protein molecular weights from 10 to 180 kDa.

Brine Shrimp Lethality Test

To measure the toxic activity of the crude venom and proteins, Brine Shrimp Lethality Test (BSLT) was used. The first step of this process was to wash 0.5 mg of *Artemia salina* eggs to remove impurities from the eggs by soaking the eggs in distilled water for 15 minutes and filtered using Whatman 125 mm filter paper. Once the eggs were cleaned, it was placed in container filled with seawater and given an aerator and a 40-watt lamp. Within 48 hours, the eggs will hatch into shrimp larvae that are ready to be tested(13). In each vial containing 1 mL of seawater, 10 shrimp larvae were placed and exposed to 1 mL samples with varying concentrations of 1, 5, 10, and 50 µg/mL by mixing the sample with seawater. Larvae that have been exposed to samples are waited for 24 hours and then manually counted for dead larvae.

DPPH Antioxidant Assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to test the antioxidant activity of the protein obtained from *S. diabolus* venom. Once the LC_{50} values from BSLT was acquired, several samples including crude venom were chosen for DPPH assay based on its its toxicity, that is samples with medium, low, and no toxic activity. Since, DPPH solution is very sensitive to light, all tubes and beakers used during the assay are ought to be covered with aluminum foil or the assay is carried out in dark place hence the DPPH solution activity does not decrease. DPPH solution was made using 2.5 mg DPPH powder dissolved in 50 mL methanol. Methanol was also used as blank, whereas control solution was made using 1 mL DPPH in 2 mL methanol. Crude venom and protein samples concentrations were varied from 1, 5, 10, and 50 µg/mL by diluting every solution using methanol, and 100 µL DPPH solution was added into 100 µL samples with varying concentrations. Incubation period of 30 minutes for samples and control solution was allowed at room temperature in dark place to complete the reactions. Using Nanodrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA), absorbance of each solutions was measured at 517 nM against blank.

Statistical Analysis

Statistical analysis was done using T-Test analysis to compare between samples.

RESULT

DNA Verification of The Fish Species

The acquired fish from Kepulauan Seribu, Indonesia, was tested to determine its specific species by using DNA verification. DNA verification process was done using DNA Barcoding method where the obtained CO1 DNA sequence is compared to the existing DNA sequence data in BLAST from NCBI website. Table I shows 5 data from BLAST analysis with the highest percentage of similarity from the fish samples obtained, as written in the %identity column. In summary, results shown in Table I indicate that the fish samples obtained were *Scorpaenopsis diabolus* species with 99.54% similarity.

Table I BLAST Analysis Result from Fish Sample

No.	Description	%Identity
1.	Scorpaenopsis diabolus voucher RH1393 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	99.54%
2.	Scorpaenopsis diabolus voucher RH1326 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	99.39%
3.	Scorpaenopsis diabolus voucher ECOMAR <FRA>:REU1031 cytochrome oxidase subunit 1 (CO1) gene, partial cds; mitochondrial	99.39%

Purification of *S. diabolus* Protein Venom

After the venom of *S. diabolus* was extracted using the batch extraction method, venom purification was carried out to assess the presence of protein contained in the venom. Figure 1 presents the result obtained from the venom purification using FPLC. Ten protein peaks (six attached peaks at 0% elution, and one peak at each 20%, 40%, 60%, and 80% elution) were produced as shown by the UV absorbance line that indicates the presence of protein in the sample at a certain elution gradient (shown by number 1 – 10 at the graph). The length of each UV absorbance peak indicates the amount of purified protein obtained, where high protein peak suggests a bigger amount of protein. Peaks that appeared at 0% elution are almost certainly due to the same charge of protein and the strong anion exchange column, therefore it was not bound to the column. Furthermore, peaks that appeared in low elution concentration might suggests that the protein eluted had a pI value nearer to the pH of the medium than the protein eluted at higher elution concentration. For the next procedures, each protein obtained from FPLC purification will be referred as peak 1 to peak 10 based on the order in which the peak appears, as written in Figure 1.

The next section of the study was concerned with the concentration of each protein peaks that was examined using the Lowry method. Standard curve equation from BSA standard absorbances was used to obtain the concentration of crude venom and proteins in each peak from purification process, as shown in Table II.

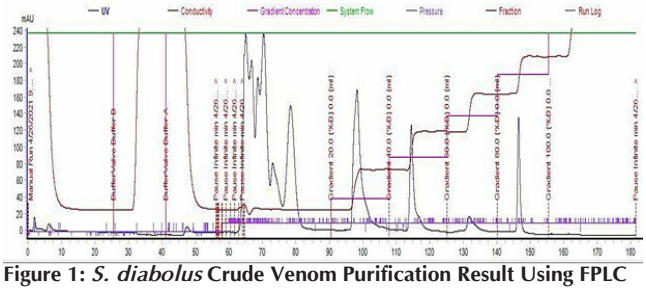

Figure 1: *S. diabolus* Crude Venom Purification Result Using FPLC

Table II Protein Samples Concentration

Sample	Concentration (µg/mL)
Crude Venom	350.81
Peak 1	112.71
Peak 2	165.10
Peak 3	174.62
Peak 4	117.48
Peak 5	122.24
Peak 6	107.95
Peak 7	169.86
Peak 8	88.90
Peak 9	69.89
Peak 10	103.19

From the table, it is apparent that the highest protein concentrations were at crude venom protein, peak 3, and peak 7 with each concentration being 350.81 µg/mL, 174.62 µg/mL, and 169.89 µg/mL respectively. Meanwhile, the lowest protein concentrations were at peaks 8, 9, and 10. The different concentrations at peaks 1 to 6 from the same elution gradient which have the possibility of being the same protein, could be due to the smaller amount of protein in the descending peaks compared to when the peaks rise due to depleted protein.

To assess a more distinct characteristic of protein, SDS-PAGE method was used to analyse molecular weights of each protein. The results obtained from the test are summarised in Figure 2 in form of protein bands. Of the ten protein samples tested, there are three protein peaks with undetectable protein bands, namely at peaks 2, 9, and 10. Contrarily, based on the Lowry test, the three peaks had a fairly high concentration, namely 165.10 µg /mL, 69.90 µg/mL, and 103.19 µg/mL respectively.

A possible explanation for this result may be due to the molecular weight of the protein that was outside the range of the protein ladder used, which was above 180 kDa or below 10 kDa. In addition, repeated freezing and thawing processess could cause protein degradation, leading to the inconsistent result. From the figure, it is apparent that crude venom well is thicker than other protein wells due to the large amount of protein contained since it was still in raw toxin form. Crude protein band result as shown in sds page result (figure 2) is different compared to other toxin crude protein as shown in other animals like stonefish, lionfish, cucumber jelly, and *Acanthaster planci* (14–17). It suggests that the crude protein from S. diabolus toxin is unique. Therefore, the observation of its potential is necessary especially the fraction of protein based on the purification in figure 1.

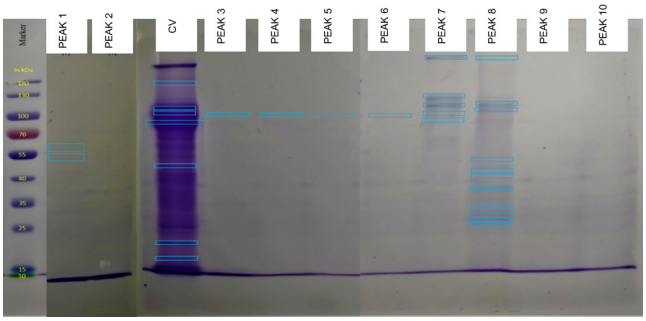

Figure 2: *S. diabolus* Crude Venom Purification Result by SDS-PAGE

Toxic Activity of *S. diabolus*

Proteins of *S. diabolus* venom was tested for its toxicity using BSLT method. Using the probit analysis method by Finney (1971), from *A. salina* larvae percentage of mortality, LC_{50} value of protein samples can be calculated with the result shown in Table III.

Table III LC_{50} Values of Protein Samples

Sample	LC_{50} (µg/mL)	T-Test (compared to Crude)
Crude Venom	10.61	n.s.
Peak 1	462.75	*
Peak 2	77.72	*
Peak 3	1.1×10^{15}	*
Peak 4	98.79	*
Peak 5	28.71	*
Peak 6	443.68	*
Peak 7	47.02	*
Peak 8	27.68	*
Peak 9	4.7×10^{11}	*
Peak 10	350.19	*

*) p<0,05; n.s.=not significant.

Compounds are considered toxic if the LC_{50} value is below 1,000 µg/mL, and are considered to have significant toxic activity if the LC_{50} value reaches 30 µg/mL or below (18). As Table III shows, there are three samples that are classified as non-toxic (LC_{50} > 1,000 µg/mL), namely the 3rd, 6th, and 9th peak protein samples. On the other hand, other eight venom samples were included in the toxic category with a significant toxic activity at crude venom, the 5th peak protein, and the 8th peak protein. Crude venom has the highest LC_{50} value because it is a raw sample that may contains more variety of toxic protein components. The high toxicity of peaks 5 and 8 might suggests that the lethal factor component of *S. diabolus* venom is present in this protein

range. Taken together, these results indicate that there is a potential of the venom to be developed further due to the low value of LC_{50} in several peaks. Low value of LC_{50} means that it has less severe effect when treated in vitro of in vivo (18). The result in this section suggests that there are protein compounds with strong, moderate and non-toxic level of toxicity found in *S. diabolus* venom.

The next section, therefore, moves on to discuss the antioxidant activity of the selected proteins with different toxicity. This selection was based on considerations to reduce the possibility of surrounding cell death if the compound is too toxic and to be safer when used for humans. Since there are no evidence yet regarding antioxidant activity from *S. diabolus* toxin extract, for this reason, the samples selected to be tested in the antioxidant activity test were crude venom, peak 1 (protein fraction 58.6 – 66.1 kDa) with LC_{50} value of 462.75 µg/mL, peak 2 (unknown protein fraction) with LC_{50} value of 77.72 µg/mL and peak 3 (protein fraction 101.1 kDa) with LC_{50} value of $1.1x10^{15}$ µg/mL. All these are selected based on toxicity level where the most toxic is peak 1 while the most non toxic is peak 3.

Antioxidant Activity of *S. diabolus*

From samples chosen based on BSLT assay, antioxidant activity of each samples was tested to determine its free radical inhibition percentage and IC_{50} value. By using DPPH method, inhibition percentage and IC_{50} values obtained can be seen in Table IV.

Table IV Inhibition Percentage and IC_{50} Values of Protein Samples

Sample	Concentration (µg/mL)	%Inhibition	IC_{50} (µg/mL)	T-Test (to Crude)
Crude Venom	1	-261.76	179.94	n.s.
	5	-123.53		
	10	-27.94		
	50	-117.65		
Peak 1	1	-250	118.31	*
	5	-213.24		
	10	-145.59		
	50	-113.24		
Peak 2	1	-102.94	64.81	*
	5	-139.71		
	10	-200		
	50	14.71		
Peak 3	1	-323.53	149.29	*
	5	-242.65		
	10	-186.77		
	50	-172.06		

* = p<0,05; n.s. = not significant

Antioxidant activity is classified as very high with an IC_{50} value of < 50 µg/mL, high at an IC_{50} value of 50-100 µg/mL, moderate at an IC_{50} value of 100-150 µg/mL, and low at an IC_{50} value of 150-200 µg/mL(19). Closer inspection of the table shows that crude venom has low antioxidant activity, peak 1 and peak 3 samples have moderate antioxidant activity, and peak 2 has strong antioxidant activity. On the other hand, a negative correlation was found between IC_{50} value and inhibition percentage, where almost all samples have negative inhibition percentage despite having small IC_{50} value, except for peak 2 with a concentration variation of 50 µg/mL. The negative value could be no antioxidant was observed from all peaks except for peak 2 at 50 µg/mL concentration and LC_{50} about 64.81 µg/mL. Interestingly, The LC_{50} of peak 2 as antioxidant is higher compared to *P. volitans* extract at 80% fractionation which is only 57.08 µg/mL (20). Further investigation is still needed to know of peak 2 antioxidant effect.

DISCUSSION

In this study, the most interesting finding was that both *S. diabolus* toxin protein and a toxin protein from another *Scorpaenopsis* species, namely *Scorpaenopsis venosa*, shares the similar molecular weight. A 33.7 kDa protein was found in peak 8 of *S. diabolus* toxin whereas a 34 kDa protein exists in *S. venosa. S. venosa* proteins with molecular weights of 71.2, 51, and 34 kDa from the venom gland was reported to have good cytolytic and hemolytic activity on goat and chicken erythrocytes, as well as a cytotoxic effect on HCT116 human colon cancer cells and SiHa cervical cancer cells(21). Accordingly, it is possible that the 33.7 kDa protein also possess the same cytolytic, hemolytic, and cytotoxic activities. However, since researches related to antioxidant testing for the *Scorpaenopsis* species has not been found elsewhere, the result of BSLT toxicity test was used as the reference for antioxidant activity testing. Another important finding was that *S. diabolus* toxin protein at peak 2 with concentration of 50 µg/mL has the percentage of free radical inhibition of 14.7%. This finding is more significant when compared to the antioxidant activity of other *Scorpaenidae* species, namely *Pterois volitans*, with 22.3% inhibition from hydrolyzed protein and 7.4% inhibition from non-hydrolyzed protein(22). Furthermore, it also have higher antioxidant activity compared to *P. volitans* extract at 80% fractionation which is only 57.08 µg/mL (20). Thus, *S. diabolus* toxin still has potential as an antioxidant compound, but some changes are needed in the antioxidant testing method.

Compared to the other fish toxins of the *Scorpaenidae* family, the antioxidant activity of *P. volitans* species is in the protein fraction with molecular weights of 7.9,

46.2, and 52.7 kDa with inhibition percentage of 5.97 – 57.08% and IC50 value of 1563.06 ppm (6). Meanwhile, in *Synanceia horrida* species, superoxide dismutase was detected with a molecular weight of ~15 kDa and thioredoxin with a molecular weight of 15 kDa(23). One possible implication of these studies is that proteins with antioxidant activity is in the range of 8 to 15 kDa. As a result, it can be estimated that the undetectable protein at peak 2 with positive antioxidant activity could be in the range of ~10 kDa and thus undetectable in the ladder protein.

The limitation of this study is the observation of antioxidant activity are done only at peak 1, 2, and 3. There are 7 peaks left that remain unobserved. Therefore, further study is required to determine whether the remaining peak have antioxidant activity. Analysis like LC/MS-MS is required to know the composition of each peak (6). Each peak could have different potential so this can explain why peak 1 and peak 3 did not show antioxidant activity. the specific molecular weight and its characterization of all proteins obtained and to test the antioxidant activity of other protein peaks.

CONCLUSION

This study has succesfully purified venom from *S. diabolus*, resulting in ten protein with varying molecular weight from 17,3 kDa to 225,1 kDa. It is also shown that there is strong antioxidant activity in the venom protein, namely in ~10 kDa protein with 50 ppm concentration. Nevertheless, this study is limited by the lack of information on the protein characterization and antioxidant activity assay on different protein peaks. Hence, further investigation should be undertaken to establish a more specific protein characterization using LC-MS/MS and also *in vitro* or *in vivo* antioxidant activity research on a broader range of purified protein from *S. diabolus* toxin.

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