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Original Article

ISOLATION OF PROTEIN FROM THE SPINE VENOM OF *PTEROIS VOLITANS* FOUND IN THE INDONESIAN OCEAN, USING A HEATING PROCESS, FOR ANTICANCER, ANTIRETROVIRAL, ANTIBACTERIAL, AND ANTIOXIDANT ASSAYS

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ABSTRACT

Objective: This research investigates the antibacterial, anticancer, antioxidant, and antiretroviral activities of the lionfish spine poison extract.

Methods: Isolation and purification of the phospholipase A2 (PLA2) protein obtained from the spine poison were conducted through the following stages, including, extraction of the venom by sonication, heating, and purification using gradual saturation levels of ammonium sulfate. Furthermore, the purity and concentration of PLA2 were analyzed using the Lowry test and Marinetti's method, respectively, while its protein content was ascertained through SDS-PAGE. Toxicity was then evaluated employing the brine shrimp lethality test (BSLT), and its anticancer activity was assessed in human cervical carcinoma cells (HeLa cells). Finally, its antioxidant, antibacterial, and antiretroviral activities were analyzed using the DPPH method, agar diffusion test against *Salmonella sp*. and *E. coli,* and SRV-2 and RT-qPCR tests, respectively.

Results: The protein demonstrated 37.79% inhibition for anticancer activity, IC_{50} 1312 ppm for antioxidant activity, 98.81%, and 89.28% inhibition of *E. coli* and *Salmonella sp*. respectively for antibacterial activity and 98.13% inhibition for antiretroviral activity.

Conclusion: It can be concluded that lionfish (*Pterois volitans*) has the potential to be developed as an antioxidant, anticancer, antibacterial, and antiretroviral agent. Furthermore, the pharmacological activity of its spine venom was determined by isolating PLA2 protein from its extract, using an optimum heating temperature of 70 °C and an ammonium sulfate saturation level of 80%.

Keywords: Pterois volitans, Crude venom, Anticancer, Antioxidant, Antibacterial, Antiretroviral

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INTRODUCTION

Pterois volitans commonly known as Lionfish are a predator species that hunts its prey such as crustaceans, small fishes, and crabs at night because it is nocturnal [1]. Although they may look unique and have an attractive shape, they sit at the top of the food chain in the ocean because their sting is a threat to other marine life-forms [2]. Reports have shown that they are capable of damaging coral reefs and altering the food chain, therefore causing imbalances in the ecosystem, which leads to population changes [3, 4]. Consequently, this fish can pose a threat to the marine life forms in countries that are close to the Indo-Pacific Ocean, which lies adjacent to the Indian Ocean, of which Indonesia is one such [5].

The thorns of lionfishes produce highly toxic venom, which on entering a victim first induces a burning sensation that lasts for about 15–20 min, then limb paralysis within 3 h [6] and sometimes cardiovascular, neuromuscular, and cytolytic effects which can lead to cell death. Moreover, its poisonous effect is abetted by the presence of toxic proteins and other active components, such as pore-forming venoms and acetylcholine [7]. Nevertheless, this poison has the potential to be used for beneficial purposes.

Another fish that happens to be related to this fish and also produces poison containing PLA2 that is believed to possess antibacterial activity is *P. russelii* [8, 9]. Moreover, the poison of lionfishes also expresses anticancer, antioxidant, and antiretroviral activities [10].

PLA2 proteins can be isolated from the poison of this fish using the extraction method as follows: A sonicator is applied to the poisons using a phosphate buffer solution [11], then the mixture is heated, and purified by precipitation using ammonium sulfate [12].

MATERIALS AND METHODS

Materials sample

The spine venom of *P. Volitans* spine found in the Indonesian Ocean was used in this research.

Sample preparation and venom extraction

The preparation of samples was carried out according to the method reported by Savitri *et al.* [12], which involved first the cutting of the spines of the lionfish under cold conditions, and then rinsing them in 0.01 M phosphate buffer (pH 7.0). 50 g of the spines were submerged in phosphate buffer (pH 7.0) containing $CaCl_2$, and an extract was prepared by sonication for 2 × 8 min with 80% pulses and an output of 10 at 20 kHz, during which the cold temperature conditions were maintained. Centrifiguration of the extract was then carried out at temperatures of 4 °C and a speed of 4500 rpm. The resultant extract was then dissolved and named 'crude venom (CV)'.

Venom protein isolation

Proteins were isolated following to procedures reported by Sommeng *et al.* [13], which involved first the heating of the CV sample at temperature variations of 50 °C, 55 °C, 60 °C, 65 °C, and 70 °C for 30 min to determine the optimum results of protein purity, and then, centrifuging it at 4500 rpm for 30 min.

Purification was then conducted following a method called ammonium sulfate fractionation. This method involved the addition of small amounts of ammonium sulfate to the pre-heated venom with the saturation of 20%, 40%, 60%, and 80%. Afterward, centrifugation was carried out for 30 min and at speeds of 4500

rpm, and the resultant sediments were submerged in a 0.01 M phosphate buffer solution (pH 7.0) which contained 0.001 M $CaCl_2$.

Determination of protein concentration

Lowry method which involved the used of both a 0.5 ml of Folin–Ciocalteu phenol 1 N Folin reagent and a biuret solution (1 ml of 1% $CuSO_4$ and NaK-tartrate solution each in 100 ml of 2% Na_2CO_3 in 0.1 N NaOH) were used to ascertain the concentration of protein contained in the sample [14]. Besides, using 200 µg/ml of bovine serum albumin (BSA), the standard curves were plotted. Lastly, the absorbance values of the sample were measured using an ultraviolet-visible (UV-VIS) spectrophotometer, at a wavelength of 750 nm.

PLA2 activity

The Marinetti method was imployed after the determination of the protein concentration in other to investigate the activity of the PLA2 protein using the enzymatic activity of the yolk of an egg [15]. Meanwhile, this as conducted because a type of lecithin in which the data is the substrate for the PLA2 enzyme or phosphatidylcholine protein is found in egg yolk. Nevertheless, using the UV-VIS spectrophotometer for 5 min and at a wavelength of 900 nm, it was observed that there was a reduction in the absorbance value without the addition of the enzyme sample.

Anticancer test using MTT assay

Through the MTT assay, using HeLa Cells, the anticancer effect of this venom sample was evaluated according to the instruction of the CellTiter 96 ° Non-Radioactive Cell Proliferation Assay [16]. Furthermore, the reagent used was Promega G400.

Dilution of both the heated (50 °C, 55 °C, 60 °C, 65 °C 70 °C) and the CV samples were conducted using PenStrep, DMEM, and 0.5% FBS solution, and the resultant samples were at concentrations of 2, 250, 500 and 2500 ppm. Furthermore, seeding of the 2×10^4 log-phase HeLa cells into 96-wells was conducted, and then incubation was carried out for 24 h before treatment with the samples of the venom. Next, the cells were incubated for another 20 h and using the MTT dye solution; they were all dyed. Following suit was another set of incubation, which lasted for 4 h before the stop solution was added. Afterward, at about 1 h later, using the ELISA reader at a wavelength of 595 nm, the absorbance values were ascertained, and all the above treatments were carried out in duplicate wells. Finally, it needs to be noted that the anticancer effect was of the venom was ascertained by calculating the rate of inhibition.

Antioxidant activity assay using DPPH

Credit goes to Sommeng *et al.* for describing the antioxidant determining method used in this research [17]. The method involved first preparing a solution of DPPH at a concentration of 125 µM by weighing 2.5 mg DPPH in 50 ml of ethanol and covering it with an aluminum foil. Next, adjusting the concentration of the solution to 20 ppm and adding 100 µl of the sample and DPPH solution to a microplate. Finally, preparing a blank using 200 µl of ethanol and covering the microplate with an aluminum foil before incubating for 30 min. The blank solution and sample were then analyzed using a microplate ELISA reader at a wavelength of 517 nm to ascertain their absorbance values, which were later plotted into the following Equation 1, to obtain the inhibition value [18]:

$$\%inhibition = \frac{blank\ absorbance - sample\ absorbance}{blank\ absorbance} \times 100\% \quad \ldots\ldots (1)$$

First, the graph of line equation was plotted from the percentage inhibition obtained for each sample, and then the resulting equation was obtained using the value x (IC_{50}) by changing the value of y = 50 [19]. All these were carried out to obtain the IC_{50} value.

Antibacterial activity test

Following the method described by Wiegand *et al.*, the venom was investigated for its antibacterial activity, using the agar dilution method, and based on the minimum inhibitory concentration [20]. Moreover, *E. coli* and *Salmonella sp* were the two species of bacterial tested.

For 24 h, the Inoculums of both bacterial were prepared to obtain a concentration of 25%. Next, they were diluted to concentrations of 10^3 CFU/ml, and the sample (both the positive (C+) and negative control (C-)) was then prepared by adding into each sterile containers, 100 µl of the inoculum.

To prepare the positive control, 10 mg of chloramphenicol was added to 10 ml to make a concentration of 1000 ppm, which was then used for both bacteria. Furthermore, the negative controls for both bacteria were prepared by adding 100 µl aq. Sterile into the Eppendorf tube. The samples (C+and C-), each at a volume of 100 µl in the Eppendorf tube were then added to the bacterial inoculum (10^3 CFU/ml) and homogenized by vortexing. Afterward, 100 µl each of the samples and controls was collected into sterile Petri dishes, and 20–25 ml of TSA was added to them. The mixture was then homogenized and incubated at 30 °C–35 °C for 24 h. Finally, the growth of Bacterial was observed, and the minimum inhibitory concentration was determined when the Petri dish was not completely overgrown with microbes. Meanwhile, the particular area of the chromatogram shows the inhibition of bacterial growth.

Antiretroviral activity test using SRV-2

To ascertain the antiretroviral activity, three tests as follows: MTT assay, RT-qPCR test, and antiretroviral activity SRV-2 test were conducted [21, 22]. Meanwhile, the MTT assay aims to ascertain how toxic the sample is to the human A549 cells (lung cancer cells) that were prepared in the Dulbecco's modified Eagle's medium (DMEM), which contained 100 µg/ml streptomycin, 5% fetal bovine serum (FBS), and 1000 U/ml penicillin.

The cells were first grown at concentrations of 5000 cells in 100 µl, and later (24 h), into them was added the extract, when the cell confluence was at 50%. Then, on the third day, to carry out the MTT test, MTT at a concentration of 5 mg/ml, and a volume10 µl was added to each well, and the mixture was incubated at 37 °C for 4 h, while the formazan crystals were dissolved in ethanol. Furthermore, after heating 20m of the sample for 20 min, 25m for 25 min, 30m for 30 min and 35m for 35 min, and adding 0.5 ml and 1.5 ml of caprylic acid (20 m, CA 0.5 ml), six types of sedimentation samples of 20% ammonium sulfate were obtained, and this test was conducted on them. Finally, the samples were tested using four concentration variations, including 1, 2, 3, and 4 ppm in triplicate.

RESULTS AND DISCUSSION

The effect of heating temperature on protein concentration

It can be seen from the results, as shown in fig. 1 that temperature is directly proportional to the concentration of the resulting protein. Therefore, with an increase in the heat, the more concentrated the protein produced is. Furthermore, fig. 2 shows the effect of temperature on the specific activity of this protein, and it suggests that the activity of the sample increases with an increase in heating time.

It was discovered the heating temperature affects protein concentration, as it increases caused a simultaneous rise in the concentration of protein produced. Therefore, it confirms that a higher heating temperature caused the isolation of more protein because the proteins which are denatured during heating get accumulated during fractionation using ammonium sulfate [13]. Furthermore, an increase in the cycles of fractionation using ammonium sulfate at higher saturation levels causes the isolation of lower concentrations of protein [23]. Lastly, it confirms that more stages of fractionation using ammonium sulfate would lead to the isolation of less protein, and this is because the maximum amount of protein could have been isolated during the previous fractionation stages using ammonium sulfate, just as it was observed with 0%–20% ammonium sulfate saturation. Meanwhile, according to Matulis (2016), ammonium sulfate fractionation stages can cause the production of protein in a more pure form [24].

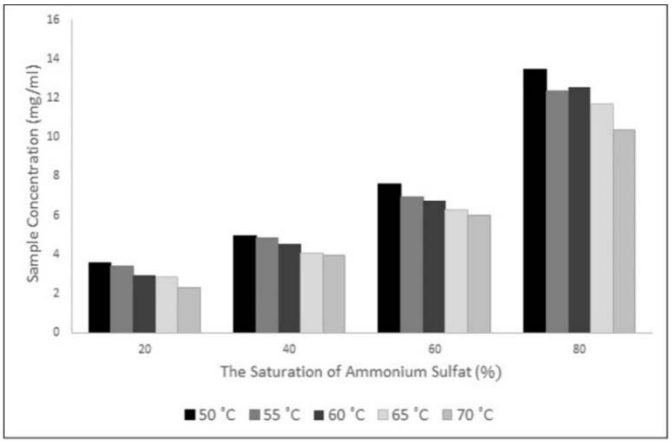

Fig. 1: The effect of heating temperature on sample concentration

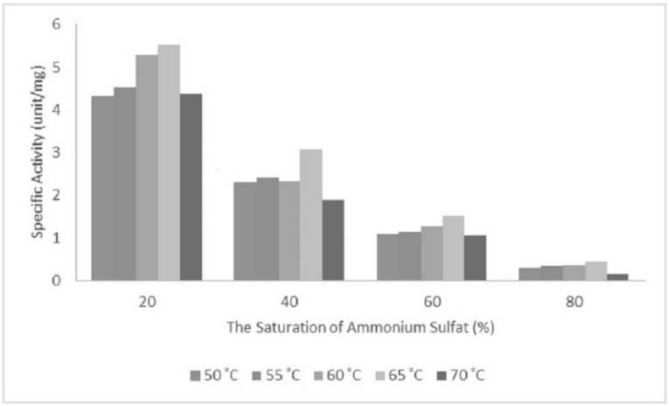

Fig. 2: The effect of heating temperature on the specific activity of PLA2

PLA2 activity was found to increase with every rise in heating temperature. Furthermore, a more effective was to obtain pure protein from the extract of Lionfish venom is through heating [16]. The CV obtained after heating at 70 °C, and fractionation using 80% ammonium sulfate, can potentially be developed into an anticancer agent since, at 750 ppm, it resulted in 37.79% inhibition. Furthermore, phospholipase A2 protein was found to be affected by the duration of heating.

Anticancer activity of *Pterois volitans* spine venom

To enhance the results, the samples were investigated for cytotoxic Anti-cervical cancer activity, using the MTT method. Moreover, according to Sommeng (2019), the values of inhibition increases with every increase in temperature and concentration [16]. Fig. 3 shows the effect of heating temperature on the inhibition of HeLa cells.

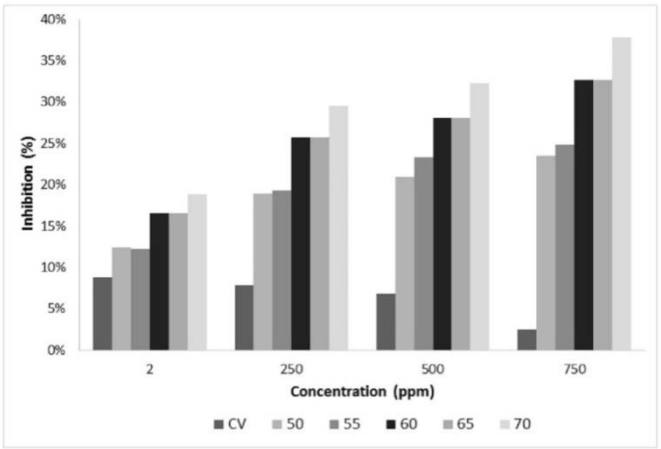

Fig. 3: The effect of heating temperature on the inhibition of HeLa cells

The investigation of the cytotoxic Anti-cervical cancer activity of PLA2 through the MTT procedure aims to ascertain the toxicity of this protein by observing whether it induces a reduction in cell viability. Furthermore, as reported in previous research by Raetz and Dowhan (1990), this procedure is colorimetric and is based on alterations in these tetrazolium salts (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), changing into formazan in the mitochondria of living cells [25]. Purple formazan crystals are then formed after the degradation of MTT, and the number formed denotes the rate of cell inhibition. Moreover, the inhibition values increase with an increase in concentration and temperature [16].

HeLa cells are affected by heating. Meanwhile, the heating process is an effective procedure used in obtaining purer protein samples from the extract of lionfish venom. The CV, which was isolated by heating at 70 °C, and fractionated using ammonium sulfate (AS) at a saturation level of 80% can potentially be developed into an anticancer agent because at 750 ppm it resulted in 37.79% inhibition. Moreover, the results of the SDS PAGE analysis showed that this fish (*P. volitans*) has several proteins which can cause an apoptotic effect on HeLa cells. This occurs when the toxin from the venom of this fish activates caspase-8, which in turn activates procaspase-3 that leads to the stimulation of caspase-3 activation [26]. The caspase cascade activation then causes the fragmentation of DNA. Meanwhile, during the apoptotic process, a specific nuclease cuts genomic DNA between the nucleosome to obtain DNA fragments, and these fragments serve as markers that help in the identification of cells that die from apoptosis [27].

Antioxidant activity of *Pterois volitans* spine venom

The number of saturation demands needed for fractionation using ammonium sulfate is influenced by several factors, one of which is the heating temperature (fig. 3). Furthermore, a temperature of 75 °C and ammonium sulfate with concentrations of 40%–60% was found to be the best conditions for this assay because they caused the highest inhibition (76.13%). Therefore, it can be suggested that proteins isolated at this temperature have the highest antioxidant activity. The IC_{50} values obtained from each sample can be seen in table 1.

Table 1: IC_{50} values obtained from each sample

Variable	IC_{50} (ppm)			
	Heating temperature (°C)			
	25 (CV)	60	75	90
AS 0%	13888.890	11627.910	4504.505	4672.897
AS 0%–20%	15625.000	8474.576	2857.143	3731.343
AS 20%–40%	10204.080	7246.377	2325.581	12820.510
AS 40%–60%	8474.576	3472.222	1312.336	38461.540
AS 60%–80%	3623.188	1742.160	6172.840	71428.710

The heating temperature affects the number of saturation requirements needed in fractionation using ammonium sulfate. Furthermore, the greatest inhibition percentage value of 76.13% was observed in samples that were heated at a temperature of 75 °C and a saturation level of 40%–60% of ammonium sulfate. This value shows that the best temperature for demonstrating the antioxidant activity of this venom is 75 °C. Furthermore, following the ammonium sulfate-grade fractionation, the most effective isolated protein was at 40%–60% ammonium sulfate saturation [17]. Therefore, a combination of the following is the best conditions for the proteins to be isolated: an ammonium sulphate saturation level of 40%–60%, and a heating temperature of 75 °C, including an IC_{50} value of 1312 ppm. Nevertheless, this IC_{50} value was the least requirement since it was obtained at>200 ppm [28]. Meanwhile, it was similar to what was obtained by Sommeng (2019), which reported a value 1563.06 ppm, which shows a greater antioxidant activity [17].

At this optimum isolation temperature, which also supports maximum antioxidant activity, the protein composition was 7.9, 46.2 and 52.7 kD. Moreover, for every change in temperature, a new saturation of ammonium sulphate is needed.

Antibacterial activity of *Pterois volitans* spine venom

It can be seen in table 2, which shows the results of the antibacterial activity test that a saturation level of AS80% is necessary for samples to inhibit bacterial growth; therefore, it can be suggested that saturation has an influence on the antibacterial activity of the samples. Meanwhile, the most potent concentration of the 35m sample was 3.77 µg/ml. Furthermore, it inhibited 98.81% of E*. coli* activity, which is very close to the LD99%, and 89.28% of *Salmonella* sp. activity, which is also very close to LD 90%. Therefore, PLA2 from the venom of lionfish can potentially be developed into an antibacterial agent.

Table 2: Antibacterial activity test results

Sample	Number of colonies of *E. coli* (CFU/100 µl)		Number of colonies of *Salmonella sp.* (CFU/100 µl)	
	I	II	I	II
AS 20%, 35m	>300	>300	>300	>300
AS 40%, 40m	>300	>300	>300	>300
AS 60%, 40m	>300	>300	>300	>300
AS 80%, 30m	116	96	317	300
AS 80%, 35m	23	48	338	305
AS 80%, 40m	142	101	321	298
K+(Chloramphenicol 1000 ppm)	0	0	0	0
K–(Sterile Aq.)	2.4×10^2	2.1×10^2	3.8×10^2	4.2×10^2

Since the samples with saturations of AS80% inhibits bacterial multiplication, it can be suggested that saturation has a role to play in antibacterial activity. Furthermore, the *E. coli* bacteria was significantly impacted when compared to *Salmonella sp.* because a large amount of phospholipid in its cell membrane is phosphatidylethanolamine, which is a preferred substrate by the PLA2, even though this enzyme is not substrate-specific [29]. Comparatively, the venom obtained from *Naja naja,* commonly known as Indian cobra, was found to be more potent at a lower concentration of 19.3 µg/ml for *E. coli* and 22.1 µg/ml for *Salmonella sp* [29].

Due to the myotoxic activities of phospholipids A2 homologs proteins, their potential antimicrobial activity has been studied [30]. Therefore, PLA2 can potentially be developed as an antibacterial agent.

Antiretroviral activity test using SRV-2

It can be seen in table 3, which shows the result of the antiretroviral activity SRV-2 test, that at concentrations of 1-4 ppm, the 20m CA 0.5 ml sample caused a significant reduction in the viral activity when compared to the negative controls. This reduction was found to be at 98.13% inhibition, which is very similar to that of the positive control where lamivudine 100 ppm was used, and which showed inhibition of 99.51%. It was also purified using the purification method, which involved salting with 20% ammonium sulfate and acidifying with 0.5 ml caprylic acid by heating at a temperature of 60 °C for 20 min. Finally, it caused a greater inhibition compared to the 30m sample, which was purified as stated above, but without acidification with caprylic acid by heating at a temperature of 60 °C for 30 min, as this showed inhibition of 88.01%.

Table 3: **Summary of preliminary test results of the isolation of phospholipase A2**

No	Sample	Copy number	%Inhibition
1	Negative Control	3,942,355	0%
2	Positive Control (Lamivudine 100 ppm)	18,973	99.51%
3	20m CA 0.5 ml 1 ppm	645,344	83.63%
4	20m CA 0.5 ml 2 ppm	533,727	86.46%
5	20m CA 0.5 ml 3 ppm	290,223	92.63%
6	20m CA 0.5 ml 4 ppm	73,593	98.13%
7	30m 1 ppm	775,363	80.33%
8	30m 2 ppm	583,224	85.20%
9	30m 3 ppm	556,011	85.59%
10	30m 4 ppm	472,334	88.01%

A greater percentage for the inhibition of Simian retrovirus serotype-2 was observed when compared to that reported by Ramadhan (2018), which showed that there was 97% inhibition using the purification method of ammonium sulfate salting gradually at a saturation level of 80% [22]. This suggests that the 20m sample has better antiretroviral activity when compared to that of lamivudine, and also, it has capabilities that are not much different from those of standard antiretroviral drugs available on the market. The potential of antiretroviral from natural product medicine must be monitored its side effects by appropriate monitoring [31].

CONCLUSION

In this research, the potential of the lionfish spine venom to be developed as an anticancer, antioxidant, antibacterial, and antiretroviral agent was determined by isolating the PLA2 protein from the venom extract. Furthermore, an optimum heating condition of 70 °C and an ammonium sulfate saturation level of 80% was used for the isolation of proteins that have the potential pharmacological activity. Lastly, the results showed 37.79% inhibition for anticancer activity, IC_{50} 1312 ppm for antioxidant activity, 98.81%, and 89.28% inhibition of *E. coli* and *Salmonella sp*. respectively for antibacterial activity and 98.13% inhibition for antiretroviral activity.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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