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First report of preparation of paralytic shellfish poisoning toxin standards from the toxic dinoflagellate *Alexandrium minutum* (Dinophyceae)

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Abstract: Paralytic Shellfish Poisoning (PSP) toxins are a group of potent neurotoxins that include saxitoxin and several structural analogues responsible for many poisoning incidents worldwide. PSP is the only harmful algal bloom (HAB) related shellfish poisoning that had been reported in Malaysia. A study has been conducted to purify the toxin compounds found in the batch cultures of *Alexandrium minutum* that is associated with PSP cases. A total of 15 batches of *A. minutum* algae cultures were extracted to determine the toxin levels using an isocratic, post-column derivatization HPLC method with fluorescence detection (HPLC-FLD). The major toxin groups detected in *A. minutum* cultures are from the GTX1&4, GTX5, and GTX2&3 groups. The highest amount of PSP toxin content detected was from batch AM07 at 7542.23 fg/cell. The culture was first purified through a Bio Gel P-2 resin to collect the fractions of the GTX1&4, GTX 5, and GTX2&3 toxin groups. The fractions of this toxin group were purified for the second time using Bio-Rex 70 resin to separate the toxin groups. Further study will be carried out to get the optimal amount of toxin. This is the first time to produce the toxin standard from the toxic dinoflagellate.

Keywords: HPLC-FLD, purification, PSP toxins, batch cultures, *Alexandrium minutum*

***Abstrak**:* Toksin Keracunan Kerang-kerangan Paralitik (PSP) adalah sekumpulan neurotoksin kuat yang termasuk saxitoxin dan beberapa analog struktur yang menyebabkan banyak kejadian keracunan di seluruh dunia. PSP adalah satu-satunya keracunan kerang-kerangan berkaitan ledakan alga berbahaya (HAB) yang telah dilaporkan di Malaysia. Satu kajian telah dijalankan untuk menulenkan sebatian toksin yang terdapat dalam kultur kumpulan Alexandrium minutum yang dikaitkan dengan kes PSP. Sebanyak 15 kumpulan kultur alga A. minutum telah diekstrak untuk menentukan tahap toksin menggunakan kaedah HPLC terbitan pasca-lajur isokratik dengan pengesanan pendarfluor (HPLC-FLD). Kumpulan toksin utama yang dikesan dalam kultur A. minutum adalah daripada kumpulan GTX1&4, GTX5 dan GTX2&3. Jumlah tertinggi kandungan toksin PSP yang dikesan adalah daripada kumpulan AM07 pada 7542.23 fg/sel. Kultur ini mula-mula ditulenkan melalui resin Bio Gel P-2 untuk mengumpul pecahan kumpulan toksin GTX1&4, GTX 5, dan GTX2&3. Pecahan kumpulan toksin ini telah ditulenkan buat kali kedua menggunakan resin Bio-Rex 70 untuk mengasingkan kumpulan toksin. Kajian lanjut akan dijalankan untuk mendapatkan jumlah toksin yang optimum. Ini merupakan kali pertama bagi menghasilkan piawaian toksin daripada dinoflagellat toksik.

Introduction

Marine biotoxins are chiefly produced by various types of toxic phytoplankton/microalgae. The proliferation of phytoplankton/microalgae producing marine biotoxins, also known as a harmful algal bloom (HAB), takes place globally. Approximately 300 species of marine microalgae are associated in harmful bloom events, of which over 100 species contain biotoxins that may cause human and animal poisoning or even death (Visciano et al., 2016). HAB has adverse environmental effects and, through bio magnifying the food web, can cause mass mortality to fish, birds, marine mammals, and human diseases by creating biotoxins that contaminate seafood (James et al., 2010; Visciano et al., 2016). The increased risk of shellfish toxicity to humans from HABs could result from large-scale ecological changes due to the anthropogenic activities, increased eutrophication, marine and aquaculture transport, and global climate change (James et al., 2010).

Several nations, including Malaysia, have reported HAB occurrences and accompanying shellfish poisoning. Paralytic shellfish poisoning (PSP), a HAB-related condition, is currently the most significant issue in this country. Dinoflagellates belonging to the genera Alexandrium, Gymnodinium, Centrodinium, and Pyrodinium generate saxitoxin (STX), a strong neurotoxin that causes PSP (Harada et al., 1982; Negri et al., 2003; Murray et al., 2012; Shin et al., 2020).

The marine dinoflagellate species Pyrodinium bahamense var. compressum, Alexandrium minutum, Alexandrium tamiyavanichii, Alexandrium taylori, and Alexandrium peruvianum have all been identified as generating PSP toxin in Malaysian seas (Usup et al., 2002a; Lim et al., 2005). The first documented PSP occurrence in Malaysia occurred in 1976 on Sabah's west coast, where approximately 201 people were poisoned including 7 of them passing away (Roy, 1977). This case was linked to Pyrodinium bahamense var. compressum (Böhm) Steidinger, Tester, and Taylor. Since that time, Sabah has experienced nearly yearly PSP incidents, and the Department of Fisheries Sabah has been regularly monitoring HAB to ensure the safety of seafood (Jipanin et al., 2019). On the other hand, Peninsular Malaysia's west and east coasts have experienced PSP since 1991. Three people were admitted to the hospital after consuming contaminated green mussels in Sebatu, Malacca in 1991 due to blooms of Alexandrium tamiyavanichii Balech (Usup et al., 2002a). One death was documented in a PSP case linked to the bloom of Alexandrium minutum located in Tumpat, Kelantan, in September 2001 (Lim and al., 2004), on which recurred in September 2015 (Lau et al., 2017). Due to the high concentration of saxitoxin in the clam tissue, the selling and collecting of shellfish from the region were outlawed (Borneo Post Online, 2015). Ten cases of PSP poisoning with the characteristic symptoms were recorded from Kuantan, Pahang, in November 2013 and again in August 2014 (Mohammad-Noor et al., 20187). The oysters had been tainted with Alexandrium tamiyavanichii. The latest case of HABs that involved PSP toxic dinoflagellate, *Alexandrium minutum* was reported in the shellfish culture area of Sg. Geting, Tumpat, Kelantan in August 2020 (unpublished).

Due to natural poisons, the PSP is one of the worst seafood poisonings. Consuming bivalve mollusks that have been exposed to marine biotoxins, such as cockles, oysters, mussels, and clams, may cause severe intoxications (Nicolas et al., 2017). This biotoxin affects the mammalian nervous system by obstructing the sodium channel, which stops the neuron signal from being transmitted. According to Backer et al. (2003), high levels of PSP can result in serious sickness and mortality from respiratory arrest within a short period of time. By ingesting toxin-producing algae, both wild and farmed shellfish get contaminated with paralytic shellfish toxins, which have been linked to PSP in humans (Watanabe et al., 2011). PSP toxins may accumulate in filter-feeding species that consume dinoflagellates, such as molluscan shellfish, and may be transferred through the trophic chain (Deeds et al., 2008). The toxins are potentially fatal to humans or other consumers, including marine mammals and birds, although they do not appear to directly affect shellfish (Huang et al., 1996). Sabah's west coast has historically been the focus of phytoplankton observation in Malaysia, but the programme has now been expanded to cover the coastal regions of Peninsular Malaysia.

There is no known antidote for PSP so far (Campbell et al., 2011). Thus, early warning by shellfish and plankton monitoring is crucial to safeguarding public health. Shellfish monitoring was carried out mainly based on the AOAC method of mouse bioassay and HPLC analysis. Reference toxins are required for both techniques. However, PSP toxins are compounds that have been labeled as chemical weapons under the Chemical Weapons Convention (CWC) and the Biological Weapons Convention (BWC), which therefore restrict their movement globally (Harju et al., 2015).

Present day, there has been relatively limited research in the development of standard material for this toxin. Commercial toxin standard is available but limited, although many countries require its control. The unavailability of toxin standards may jeopardise our seafood monitoring program, thus compromising our consumers to possible poisoning and jeopardising our export to many countries. Therefore, setting up an in-country production of the standard biotoxin capability is a critical step in ensuring the success of our seafood monitoring program. This study aimed to isolate and purify the

toxin compounds detected in the culture of *Alexandrium minutum* that is associated with PSP cases by several types of chromatography. HPLC then determined the purified toxins.

Materials and Methods

Chemicals and Standards

All of the solvents were HPLC-grade. Methanol, acetic acid, and acetonitrile of HPLC grade were purchased from J.T. Baker, Avantor, USA. Octanesulfonic acid and tetrahydrofuran were acquired from Fisher Chemical and Sigma, respectively, in the USA. Other substances were of an analytical quality. An Ultra-Pure Water System (Evoqua Water Technologies, Germany) was utilised to clean the water for HPLC.

PSP Toxin Standards

Standard stock solutions of individual PSP toxins (Saxitoxin (STX), decarbamoylsaxitoxin (dcSTX) and gonyautoxin-5 (GTX5)) and standard stock solutions of mixed PSP toxins (gonyautoxin-1&4 (GTX1&4), gonyautoxin-2&3 (GTX2&3)) (NRC, Canada, Halifax) were purchased from the Groupe Biomedix Sdn Bhd. Selangor (except C-toxins, which were temporarily unavailable).

Culture of A. minutum

The pure culture in this study was obtained from Universiti Kebangsaan Malaysia (UKM), which was isolated during the PSP case in Geting, Tumpat, Kelantan in 2001. This species was confirmed to produce GTX4, GTX1 toxins up to 90 % of the toxin composition (Lim et al., 2007). Cultures were maintained in 10 L medium ES-DK (Kokinos and Anderson, 1995) at 15 ppt salinity, the temperature was maintained at 25 ºC under a light intensity of 70 μmo photon/m²/s below 16:8 h light:dark photocycle until the culture reaches the exponential growth phase (> 20,000 cells/mL). Phytoplankton cell counts were performed using the Sedgwick Counter Chamber under the observation of the Inverted Microscope Olympus IX51 (Olympus, Japan).

PSP toxin extraction

The method of centrifugation was utilized to harvest the Alexandrium cultures for the toxin analysis (Eppendorf 5430, Hamburg, Germany). The cells were lysed using an ultrasonic homogenizer (OMNI-Ruptor 4000, Georgia, USA) following the process where the cell pellet was resuspended in 0.05 M acetic acid. The supernatant was then collected after the sample had been centrifuged at 10,000 g for 10 minutes. By running the extract through a 0.45 m nylon filter, the extract was cleaned thoroughly. Before further research, the extracted supernatant from the collected sample was kept at -20 °C. Toxin content was evaluated using HPLC.

PSP toxin analysis

The analysis of the toxins was executed using HPLC (Shimadzu, Japan) equipped with a Pickering post-column device and fluorescence detector utilising the isocratic, post-column derivatization with slight modifications method of Oshima (1995). The resulting samples were then separated using a security guard cartridge (C18, 4 x 3.0 mm inner diameter) and a Luna C18(2) column (150 x 4.6 mm inner diameter, 120, 5 m) from Phenomenex in Torrance, USA, at a flow rate of 0.8 mL/min. The post-column temperature was set for roughly 65 °C for all runs while the column temperature was maintained at 27 °C. By substituting distilled water for the oxidising reagent, toxin verification was carried out in non-oxidizing post-column conditions. The reaction coil was kept in a cold bath during the analysis. The mobile phase for the STXs was 2 mM heptanesulfonate in a buffer solution of 30 mM ammonium phosphate and 5% acetonitrile (v/v), pH 7.1, and for the GTXs, it was 2 mM heptanesulfonate in a solution of 10 mM ammonium phosphate and 1% acetonitrile (v/v), pH 7.1. The post-column oxidising agent was 7 mM periodic acid in 10 mM sodium phosphate buffer, pH 9.0, and the acidifier was 0.5 M acetic acid. The sample injection volume for each run was 10 L, and the post column flow rate was 0.4 mL/min. Excitation and emission detection wavelengths were selected at 330 nm and 390 nm, respectively. Each sample was examined three times. Separate analyses of GTXs (GTX 1–5) and STXs (STX, dcSTX) were conducted. By comparing the identified toxin to standard

toxin materials, it was possible to identify and quantify the toxin. Each toxin's or epimeric pair's concentrations (GTX1&4, GTX2&3, GTX5, STX, and dcSTX) were determined using linear calibration curves that were created using PSP-certified reference standards. The following values of the toxicity factor translate to STXequiv. were found for the calculation of toxicity from HPLC chromatograms: GTX1 (0.99), GTX2 (0.36), GTX3 (0.64), GTX4 (0.73), GTX5 (0.06), C1 (0.01), C2 (0.1), dcSTX (0.51), NEO (0.92), and STX (1).

Toxin purification

Accompanied with slight adjustments, the procedure outlined by Laycock et al. (1994) was used to discover how to purify GTXs from the sample extracts. A Bio-Gel P-2 column (fine; 25 mm x 200 mm; BioRad, Hercules, CA, USA) and a Bio-Rex 70 (20 - 50 mesh; 30 mm x 300 mm; BioRad, Hercules, CA, USA) were used to purify the extracts. These columns were equilibrated with deionized distilled water (Evoqua Water Technologies, Germany). The sample was loaded onto the column and eluted with 0.1 M AcOH at a flow rate of 10 mL/min. The Flash Chromatography Purification System (Teledyne ISCO, USA) was used for the collection of each ten-milliliter fraction. Further analysis and quantification of each fraction was done using HPLC. Fractions of each compound were combined, lyophilized, and then dissolved in 0.05 M AcOH. For further study, the samples were held frozen at -20 °C.

Statistical analysis

The statistical programme Statistical Package for Social Sciences (SPSS) version 16.0 for Windows was used to evaluate data on PSP concentrations. After performing a one-way analysis of variance (ANOVA) on the toxicity data to assess variations in the mean PSP of various batches of the A. minutum culture, Tukey's post hoc test analysis was performed. When P 0.05, means and standard deviations of values made in triplicate were reported and deemed substantially different.

Results and Discussion

In Malaysia, paralytic shellfish poisoning cases have been linked to many Alexandrium species (Usup et al., 2002b). HPLC toxin analysis showed that this sample's PSP toxins were created by A. minutum culture. The GTX group's chromatogram is displayed in Figure 1. A. minutum has been found to include the following primary toxin groups: GTX1, GTX2, GTX3, GTX4, and GTX5, with GTX1 and GTX4 being the dominating derivative (Figure 1a & b). In this order, the toxins were eluted: GTX4, GTX1, GTX5, GTX3, and GTX2. By comparing these toxins to the GTXs standard, it was determined that they belonged to the GTXs group (Figure 1c). Usup et al. (2006) also shown that only GTX1, GTX2, GTX3, and GTX4 were present in A. minutum. A. minutum only produced GTX 1 and 4, with GTX 4 being the dominant derivative, according to the majority of investigations conducted to date (Hwang and Lu, 2000; Yoshida et al., 2000; Lim et al., 2004). However, distinct toxin profiles have been discovered for some isolates, including neosaxitoxin (NEO) and sulfocarbamoyl toxins (C-toxins) produced by isolates from Denmark and New Zealand (Hansen et al., 2003) and New Zealand (Chang and McClean, 1997).

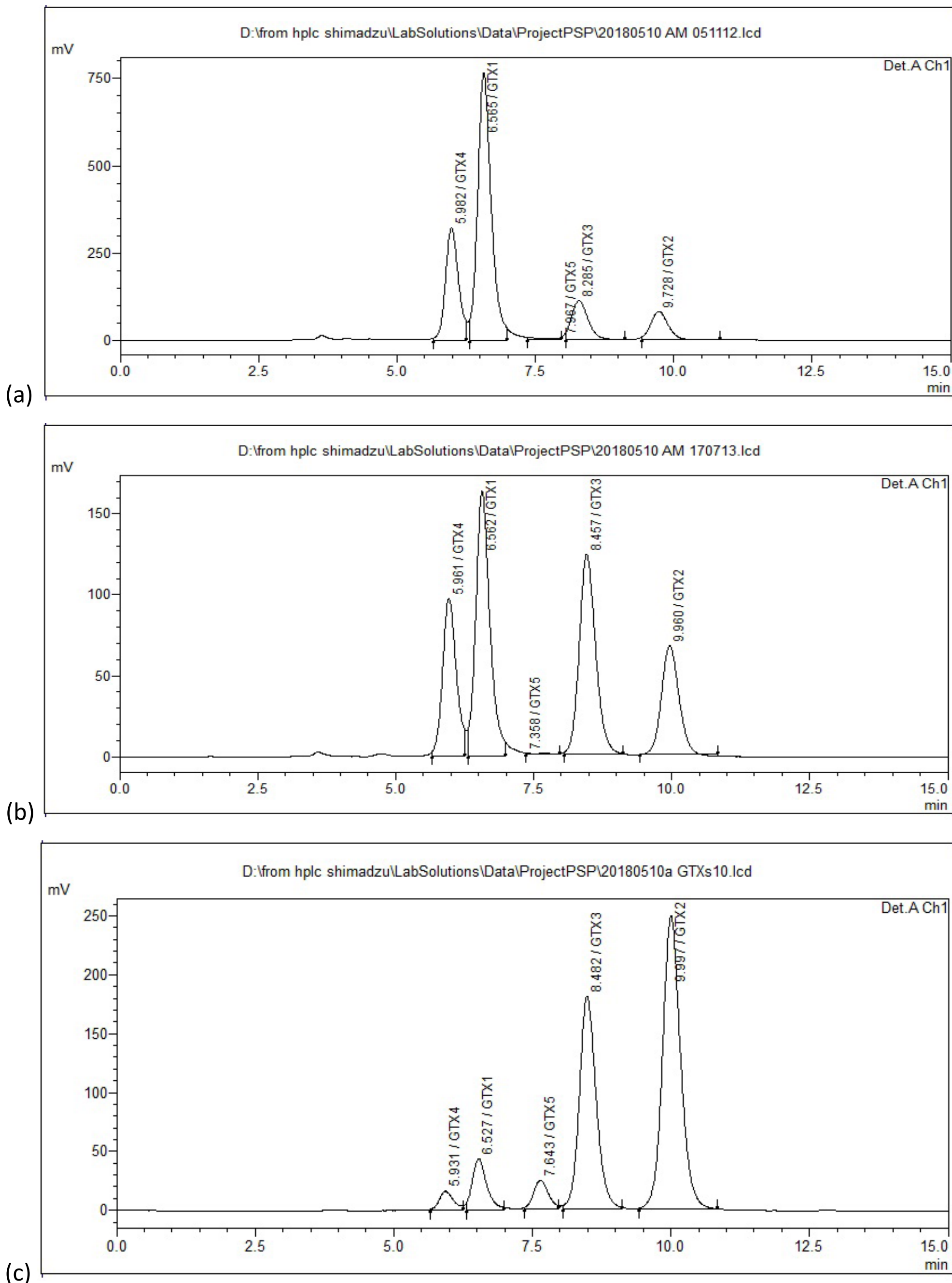

Figure 1. Toxin profiles of *A. minutum* from batch AM10 (a), batch AM09 (b) and GTXs standard (c).

A total of 15 batches of *A. minutum* cultures were extracted to determine the toxin levels in this study. There was a significant difference in mean total PSP (fg/cell) between the batches ($P < 0.05$). The highest cell toxin content was found from the batch AM07 at 7542.23 fg/cell followed by batch AM06 and batch AM08 with the mean total PSP of 4377.35 fg/cell and 4116.23 fg/cell, respectively (Figure 2). It has been shown that the toxin content of a cell varied widely depending on growth conditions (Usup et al., 1994; Cembella, 1998). Analysis showed that culture of *A. minutum*

from batch AM07 contained an average of 4569.79 fg/cell GTX4, 2733.74 fg/cell GTX1, 71.87 fg/cell GTX5, 110.03 fg/cell GTX3 and 56.79 f fg/cell GTX2. The other three toxins were absent in this culture (Figure 3).

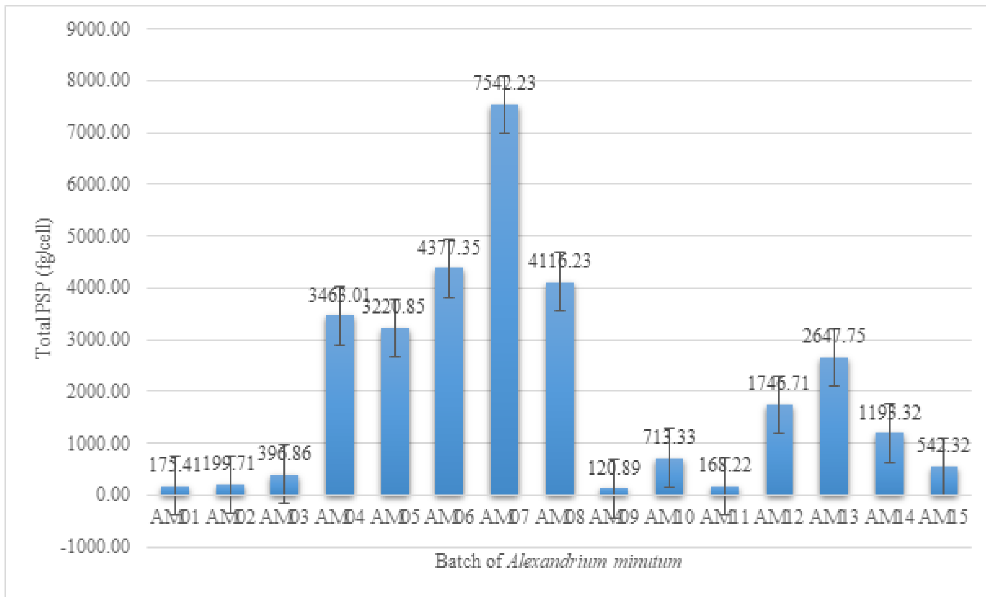

Figure 2. Mean of total PSP concentration (fg/cell) from different batch cultures of *A. minutum*.

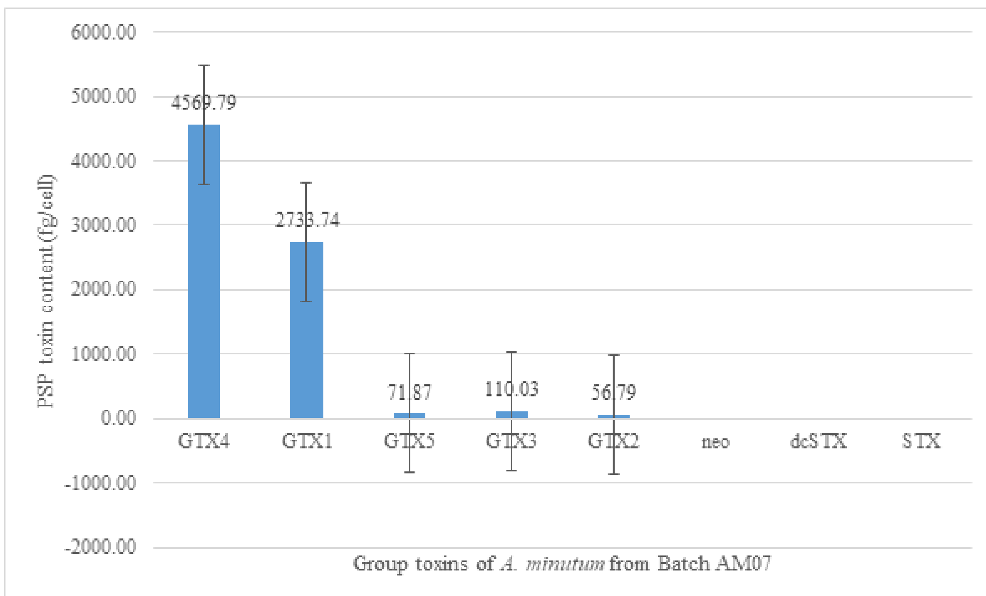

Figure 3. Mean of PSP toxins (fg/cell) in the culture of *A. minutum*.

The toxin obtained from each batch culture of *A. minutum* was subjected to Bio Gel P-2 column chromatography equilibrated with distilled water. The majority of other soluble small compounds in the extract can be effectively removed using this procedure. By using chromatography on Bio Gel P-2, it is necessary to carefully separate toxins with net charges of zero or negative integers. On the

column, the toxin was almost entirely absorbed. The toxin was then eluted with 0.03 M acetic acid after the column had been thoroughly cleaned with distilled water. Figure 4 displays an illustration of the elution profile from the batch culture of A. minutum.

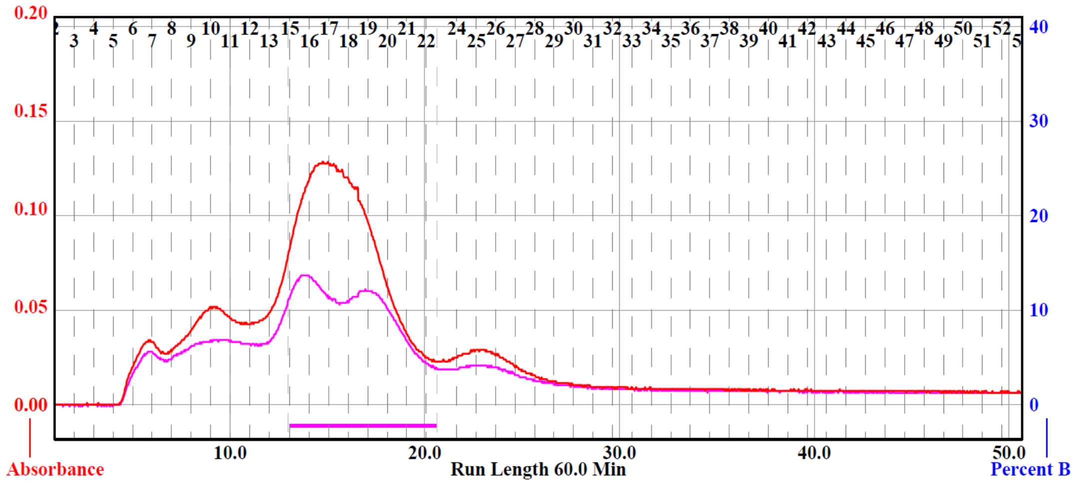

Figure 4. Elution profile of *A. minutum* culture from Bio Gel P2 column (2.5 x 20 cm)

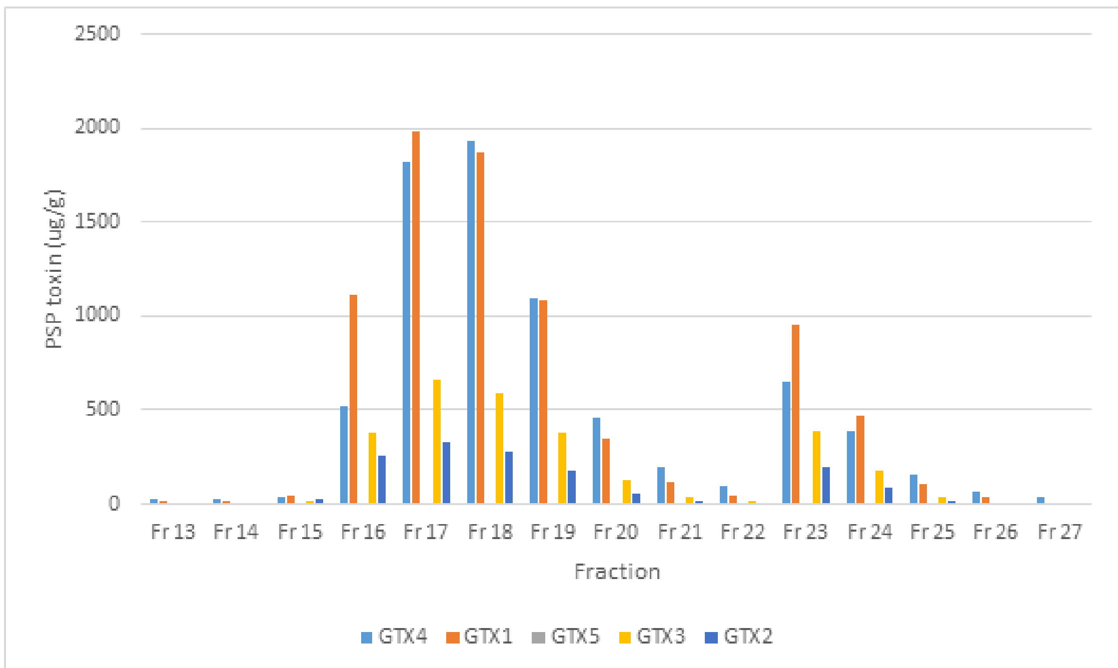

Figure 5. The content of PSP toxin groups from the batch of *A. minutum* culture after purification with Bio Gel P2 resin.

Tube from fraction 5 to fraction 28 were collected and analysed individually by HPLC. Analysis of the toxic fractions by HPLC showed that GTXs group was detected from fraction 16 to fraction 20 and fraction 23 to fraction 25. The range of total amount of GTXs in these fractions was 24.79 – 1931.18 µg/g (GTX4), 10.63 – 1986.85 µg/g (GTX1), 0 – 4.03 µg/g (GTX5), 2.73 – 659.24 µg/g

(GTX3) and 0.59 – 328.45 µg/g (GTX2). The toxic fractions were combined, concentrated and lyophilized. The toxins were further purified on a Bio-Rex 70 column with linear concentration gradients of acetic acid from zero to 3 M using 500 mL. However, no fraction was detected to contain GTXs group after purified by Bio-Rex 70 column. Figure 6 shows that the separation on Bio-Rex 70 was not complete for any of the five toxins. This might be due to the toxin was not adsorbed on cation-exchange resin (Bio-Rex 70) because of high concentration of acetic acid or the column was not packed properly. Several alternative adsorptions were tried without significant improvement in resolving the toxins. For the Bio Gel P-2 column, resolution improved with column length and small sample volumes (Laycock et al., 1994).

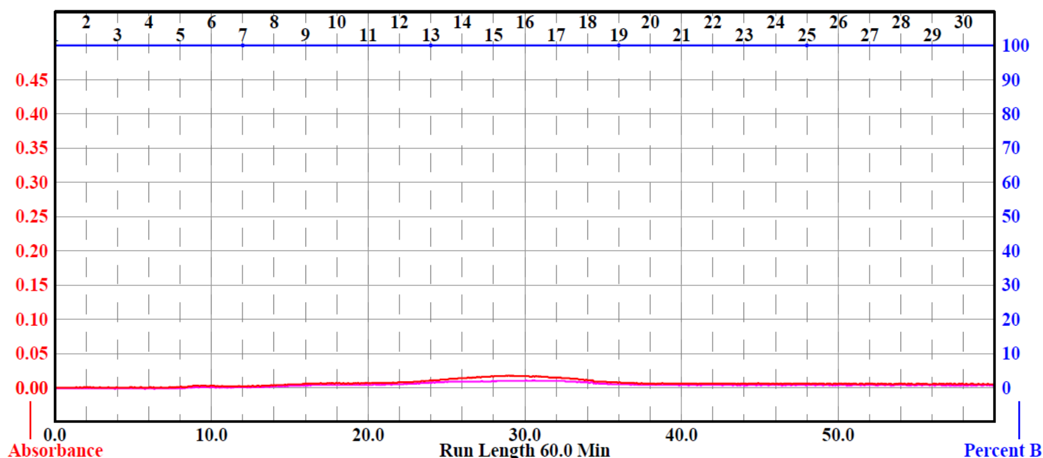

Figure 6. Elution profile of *A. minutum* culture from Bio-Rex 70 column (2.5 x 20 cm)

Conclusion

In the present study conducted, the main toxin groups identified in the current study's cultures of A. minutum were GTX1, GTX2, GTX3, GTX4, and GTX5, with GTX1 and GTX4 producing the dominating derivative. The results showed that the purified toxin from the *A. minutum* contained 5.56 µg/g (GTX1), 27.41 µg/g (GTX4), 1441.59 µg/g (GTX5), 1025.72 µg/g (GTX2) and 42.13 µg/g (GTX1) after purification by Bio Gel P-2 column chromatography. Nevertheless, further purification on Bio-Rex 70 was not accomplished for any of the five toxins. Further study will be carried out to gain the optimal condition on Bio-Rex 70.

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