TẠP CHÍ KHOA HỌC
TRƯỜNG ĐẠI HỌC QUY NHƠN

Biến động theo mùa, trọng lượng và giới tính của độc tố tetrodotoxin trong cua móng ngựa *Carcinoscorpius rotundicauda* thu từ biển Cần Giờ, thành phố Hồ Chí Minh, Việt Nam

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TÓM TẮT

Biến động của hàm lượng tetrodotoxin (TTX) trong cua móng ngựa *Carcinoscorpius rotundicauda* được thu thập từ bờ biển Cần Giờ, Thành phố Hồ Chí Minh, Việt Nam từ tháng 5/2021 đến tháng 5/2022 theo mùa, trọng lượng cơ thể và giới tính được xác định bởi phương pháp sắc ký lỏng kết hợp khối phổ (LC-MS/MS). Dữ liệu cho thấy có sự thay đổi đáng kể về hàm lượng TTX theo tháng với độc tính TTX tối đa được xác định là 197,6 ± 134,5 MU/g đối với các mẫu được thu thập vào tháng 5 năm 2021, trong khi *C. rotundicauda* được thu thập vào tháng 1 năm 2021 chỉ đạt hàm lượng TTX trung bình 7,8 ± 8,4 MU/g. Điều thú vị là sự khác biệt về hàm lượng TTX ở các nhóm *C. rotundicauda* trọng lượng cơ thể khác nhau cũng có khác biệt về mặt thống kê, với độc tính TTX tối đa là 230,3 ± 116,3 MU/g được xác định cho các mẫu vật có trọng lượng cơ thể lớn hơn 300g, trong khi mức TTX thấp nhất (9,8 ± 12,3 MU/g) được đo cho nhóm *C. rotundicauda* có trọng lượng cơ thể dưới 150g. Hơn nữa, có sự khác biệt đáng kể về hàm lượng TTX giữa các nhóm *C. rotundicauda* cái và đực, với độc tính TTX cao hơn đáng kể được xác định cho mẫu cua móng ngựa cái (123,9 ± 45,8 MU/g) so với *C. rotundicauda* đực (68,7 ± 45,8 MU/g). Đặc biệt, hàm lượng TTX trung bình trong các mẫu cua móng ngựa thu ở biển Cần Giờ đạt 96,3 ± 94,2 MU/g là khá cao, cho thấy chúng không an toàn cho con người sử dụng làm nguồn thức ăn. Tuy nhiên, cua móng ngựa từ vùng biển Cần Giờ, thành phố Hồ Chí Minh, Việt Nam là nguồn tiềm năng để phân lập và tinh sạch tetrodotoxin cho các ứng dụng khác nhau (ví dụ làm thuốc).

Từ khóa: *Carcinoscorpius rotundicauda, Tetrodotoxin (TTX), LC-MS/MS.*

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Seasonal, body weight and gender variation of tetrodotoxin in horseshoe crab *Carcinoscorpius rotundicauda* collected from Can Gio coast, Ho Chi Minh city, Vietnam

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ABSTRACT

Liquid chromatography-mass spectrometry (LC-MS/MS) was used for measurement of the variation of tetrodotoxin (TTX) in a horseshoe crab *Carcinoscorpius rotundicauda* collected from Can Gio coast, Ho Chi Minh City, Vietnam from May 2021 to May 2022 over the seasons, body weight and gender. The data revealed that there was significant variation of TTXs level over the months with maximal TTXs toxicity determined of 197.6 ± 134.5 MU/g for specimens collected in May 2021, whereas the *C. rotundicauda* collected in January 2021 was found to yield minimum TTXs level of 7.8 ± 8.4 MU/g. Interestingly, the difference of TTXs level in different body-weight *C. rotundicauda* groups was also statistically significant, with maximal TTXs toxicity of 230.3 ± 116.3 MU/g determined for specimens having body weight of greater than 300g, while the lowest level of TTXs (9.8 ± 12.3 MU/g) was assayed for *C. rotundicauda* group of body weight less than 150g. Moreover, there was a significant difference in TTXs levels among female and male *C. rotundicauda* groups, with notable higher TTXs toxicity determined for female specimens (123.9 ± 45.8 MU/g) than that of male *C. rotundicauda* (68.7 ± 45.8 MU/g). In particular, the average level of TTXs of 96.3 ± 94.2 MU/g measured in all studied crabs indicated that they are unsafe for human consumption. Fortunately, horseshoe crab collected from Can Gio coast, Ho Chi Minh City, Vietnam is a promising source for isolation and purification of tetrodotoxin for other applications (e.g., drug).

1. INTRODUCTION

Among four horseshoe crab species often found in Asia region,[1,2] *Cacinoscopius rotundicauda* is reported as poisonous, and intoxication due to its intake. Previous studies have been reported that *C. rotundicauda* generally contains majority of tetrodotoxin (TTX) and minor amount of paralytic poisoning (PSP) toxins.[1-3] These toxins have caused widespread of food poisoning due to consumption of horseshoe crabs in Thailand,[4-8] Malaysia,[9] China,[10,11] and Vietnam.[1] It was evaluated that the mortality caused by consumption of *C. rotundicauda* in Thailand was about 1.75%, which was considerable high.[12] Consequently, identification and determination of toxin and toxicity in *C. rotundicauda* in Asian countries is critically important for examining potential hazard of this creature when they are

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often consumed as one of seafood. Although several studies have been carried out to determine toxin and toxicity of *C. rotundicauda* collected from China, Thailand, Cambodia, Malaysia and Vietnam, these studies were case-by-case investigation with specimens collected in one time only. Remarkably, it has generally stated that Thai people can be poisoned by *C. rotundicauda* at certain seasons of the year, with peaked number of poisoning cases between December and March.[6,7] However, the authors did not determine variation of toxins and toxicity of *C. rotundicauda* collected over the months of year. Moreover, Ngy et al.[13] only investigated variation of TTXs in *C. rotundicauda* collected from Cambodia within two successive months during rainy (April-May) and dry (December-January) season. In mouse-based experiments, Liao and Li summarized that the toxicity of adult horseshoe crabs was much higher than that of young group without quantification of toxin and toxicity. Furthermore, majority of studies discussed that toxicity dominantly accumulated in eggs of female horseshoe crabs.[3,13,14] Nevertheless, statistical evaluation of the variation of toxin and toxicity levels in *C. rotundicauda* collected over different collection times, body weight and gender was not reported in the literature.

In this work, *C. rotundicauda* collected from Can Gio coast, Ho Chi Minh city, Vietnam from May 2021 to March 2022 with the total of six collections (60 specimens) analyzed for examining the variation of toxicity and toxin over seasonal variation climate in Vietnam. Extensively, the sixty specimens were divided into five groups based on body weight of *C. rotundicauda* and two groups of male *C. rotundicauda* and female *C. rotundicauda* for statistical analysis of toxin composition and toxicity level. The data obtained in this work will alert Vietnamese citizens about potential hazards when using *C. rotundicauda* as a seafood. More importantly, the results reported in this study will point out that *C. rotundicauda* is a promising source for isolation and purification of tetrodotoxin which is currently applied as a bioactive compound for drug formulation (e.g., cancer treatment, pain treatment, etc.).

2. MATERIALS AND METHODS

2.1. Chemicals

Tetrodotoxin (TTX, 4-epiTTX, Anh-TTX), formic acid and acetic were obtained from Wako pure chemicals (Osaka, Japan). Saxitoxin (C1/2, GTX1-5, dcGTX2/3, NEO, dcSTX) was a gift from Dr. Oshima, Tohoku University, Japan. Ammonium hydroxide 25% for liquid chromatography-mass spectrometry (LC-MS) was purchased from Sigma-Aldrich (Tokyo, Japan). Acetonitrile was purchased from Kanto Chemicals (Tokyo, Japan).

2.2. Specimen collection

Horseshoe crabs *C. rotundicauda* were collected from Can Gio coast, Ho Chi Minh City, Vietnam from May 2021 to May 2022. The specimens were classified by referencing relevant literature.[1,3] After transferred to the Laboratory of Technology of Bioactive Compounds, Institute of Chemistry, Vietnam Academy of Science and Technology, specimens were immediately frozen and kept frozen at −20°C for sample preparation and toxin extraction.

2.3. Extraction and analyzing of tetrodotoxin and saxitoxins

Specimens were dissected to collect all soft tissues, which were then homogenized with acetic acid 1% (2:1 w/v). The homogenized samples were boiled for 5 min, followed by cooling down at room temperature and centrifugation at 9,000 rpm, 20 ^{o}C for 10 min to collect supernatant. The supernatant was treated using an ENVI-Carb SPE cartridge 250 mg/3 ml (Sigma Aldrich Japan, Tokyo, Japan), eluted by four-fold diluted acetonitrile (MeCN) with 1% acetic acid. The TTXs-rich eluate was analyzed by a Hydrophilic interaction liquid

Chromatography-Mass spectrometer (HILIC/MS-MS) coupled to a Shimadzu system triple-quadrupole mass spectrometer (LCMS−8040; Shimadzu Corporation, Kyoto, Japan). The HILIC separation was performed using a Waters Xbrige (HILIC) Amide column (4.6 mm I.D ×150 mm, 3.5 µm) at 60°C with 5 µl sample volume injected. Mobile phases were water/formic acid/ammonium hydroxide (500:0.075:0.3 v/v/v) (A); acetonitrile/water/formic acid (700:300:0.1 v/v/v) (B) with flow rate of 0.6 mL/min.

The chromatographic conditions consist of initial conditions 100% B, held for 20 min, then a linear gradient 50:50 A and B within 15 min, held for 9.90 min. Ion source parameters of MS spectrometer were as follows: Entrance Potential (EP): 10 V; Curtain gas (CUR): 30 psi; Ion Spray Voltage (IS): 4500 V; Source desolvation temperature (TEM): 250 ºC; Source ion block temperature: 400 °C; Desolvation gas flow: 1000 L/h, Nebulizer gas flow: 2L/min; Collision gas flow rate: 0.15 mL/min. Multiple reaction monitoring (MRM) was performed in positive electrospray ionization (ESI^+). A minimum of two transitions were used for each TTX and STX analogues. For each target ion, an MRM ion channels were selected for specific product ions generated from the selected precursor ion.[15] To confirm TTXs in the extract of horsecrabs, MS/MS spectra were obtained at -25 eV of collision energy with m/z 320.1 > 302.1 for TTX and 4epi-TTX, m/z 302.0 > 284.1 for Anh- TTX in the 1st transition; and -40 eV with m/z 320.1 > 162.1 for TTX and 4epi-TTX, m/z 302.0 > 162.0 for Anh-TTX in the 2nd transition. STXs were scanned in the same mode with mass ranging from m/z 50-m/z 350.[16] Toxicities were calculated from HILIC-MS/MS data and expressed in mouse unit (MU/g) according to Nakamura and Yasumoto (1985),[17] in which 1 mg TTX corresponding to 4500 MU, 4-epiTTX to 710 MU and 4,9-anhydroTTX to 92 MU. One MU is the dose of toxin that will kill a 20 g male mouse (ddY) in 30 min.

2.4. Statistical analysis

Experiment was carried out in triplicate and data was reported as mean ± standard error (SE). Statistical analysis was done using one-way ANOVA followed by post hoc Tukey's test and a *p*-value of < 0.05 was declared as significant. The statistical analysis was conducted using software package IBM SPSS statistics (SPSS 22, SPSS Inc., IBM, New York, USA).

3. RESULT AND DISCUSSION

3.1. Seasonal variation of tetrodotoxin

Horseshoe crab specimens were all identified as *Carcinoscorpius rotundicauda*.[1,3] The toxicities of all the specimens were calculated based on the specific toxicity of each toxin component and expressed in mouse units (MU) (Table 1).[18]

Table 1. TTX toxicity (MU/g) of *Carcinoscorpius rotundicauda* specimens collected from Can Gio coast, Ho Chi Minh city, Vietnam in 2021 - 2022.

Specimen No.	Body size			Toxicity (MU/g)			Total toxicity (MU/g)
	Weight (g)	Length (cm)	Width (cm)	4epi-TTX	TTX	Anh-TTX	TTXs
May 2021							
f1	300.1	34.1	18.3	11.7±0.13	56.2±2.72	49.8±2.73	117.6±5.09
f2	210.3	24.5	12.5	7.9±0.09	58.5±3.04	10.7±0.72	77.1±3.78
f3	305.2	33.0	18.0	18.9±1.21	304.1±9.23	4.8±0.11	327.8±13.36
f4	307.1	33.5	17.2	1.4±0.04	331.4±10.32	3.7±0.06	336.5±11.14
f5	319.4	35.2	19.5	17.4±1.04	424.1±12.27	39.1±1.33	480.6±19.34

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Specimen No.	Body size			Toxicity (MU/g)			Total toxicity (MU/g)
	Weight (g)	Length (cm)	Width (cm)	4epi-TTX	TTX	Anh-TTX	TTXs
m1	290.5	32.5	16.7	105.4±3.72	28.9±0.55	2.2±0.05	136.5±5.81
m2	230.1	29.5	12.5	68.6±2.88	41.3±1.17	4.0±0.62	113.9±9.63
m3	251.6	25.5	15.2	56.8±1.23	49.2±2.16	5.8±0.22	111.7±8.77
m4	261.2	24.5	18.5	6.9±0.03	109.1±5.22	12.5±0.72	128.5±7.82
m5	215.4	26.6	11.2	45.3±1.82	98.5±4.06	1.8±0.04	145.5±10.77
October 2021							
f6	278.1	27.5	11.8	67.1±2.08	56.4±1.26	33.3±0.52	156.8±13.16
f7	311.2	34.5	17.5	40.0±1.13	39.8±2.67	42.0±1.88	121.8±10.66
f8	315.0	34.5	19.5	16.3±0.71	48.0±3.11	52.0±2.89	116.4±7.62
f9	227.1	28.5	15.2	27.0±1.52	39.5±0.97	47.0±2.89	113.5±7.25
f10	177.3	23.6	14.4	13.1±0.24	48.8±2.72	2.0±0.04	63.8±2.81
m6	195.5	25.5	16.6	7.1±0.11	40.9±1.22	28.0±2.90	76.0±3.78
m7	159.1	27.6	15.7	6.6±0.08	31.2±0.97	19.9±0.62	57.6±1.98
m8	197.6	28.0	12.0	17.0±1.26	47.7±3.10	10.7±0.47	75.5±3.38
m9	267.1	32.5	17.5	18.7±1.02	42.8±3.67	32.1±1.88	93.6±2.09
m10	295.3	33.5	18.5	28.9±1.08	40.2±1.89	21.6±0.71	90.6±4.78
November 2021							
f11	100.3	21.0	11.0	1.7±0.05	1.2±0.04	0.3±0.01	3.1±0.09
f12	210.1	29.0	13.0	5.6±0.17	86.8±2.60	1.2±0.05	93.6±3.81
f13	104.6	25.6	15.5	1.2±0.04	3.0±0.05	1.0±0.03	5.2±0.16
f14	182.0	26.6	12.1	7.6±0.23	2.0±0.06	0.1±0.01	9.7±0.29
f15	115.1	25.2	15.3	4.0±0.12	2.0±0.09	0.7±0.02	6.8±0.22
m11	250.3	29.5	15.4	51.3±2.54	37.0±1.13	11.1±0.33	99.4±2.78
m12	104.5	22.4	15.4	1.6±0.03	3.0±0.07	0.4±0.01	5.0±0.13
m13	259.2	30.6	17.5	12.8±0.37	22.7±0.68	1.2±0.04	36.7±1.12
m14	272.3	31.6	18.5	14.9±0.45	48.6±1.46	1.3±0.05	64.8±1.94
m15	165.1	23.5	14.3	9.4±0.28	35.9±1.08	29.6±0.78	75.0±2.56
January 2022							
f16	104.0	25.0	11.0	0.0±0.00	0.0±0.00	2.1±0.06	2.1±0.02
f17	110.4	25.7	13.0	0.6±0.02	4.1±0.11	0.1±0.00	4.8±0.11
f18	109.2	26.1	15.4	2.1±0.06	2.9±0.08	0.1±0.00	5.1±0.15
f19	112.1	24.9	11.5	0.9±0.02	3.0±0.08	1.1±0.03	5.1±0.17
f20	168.3	22.5	11.0	15.7±0.42	11.7±0.32	3.6±0.10	31.0±0.04

Specimen No.	Body size			Toxicity (MU/g)			Total toxicity (MU/g)
	Weight (g)	Length (cm)	Width (cm)	4epi-TTX	TTX	Anh-TTX	TTXs
m16	101.1	25.2	12.1	0.9±0.02	2.3±0.06	1.0±0.03	4.2±0.11
m17	213.3	32.0	13.5	6.0±0.16	1.9±0.05	1.1±0.02	8.9±0.24
m18	109.5	23.0	10.0	1.0±0.03	3.2±0.09	0.1±0.00	4.3±0.12
m19	248.1	30.0	15.0	1.2±0.03	2.8±0.08	3.7±0.10	7.7±0.18
m20	210.2	29.0	13.0	1.5±0.04	2.3±0.06	1.3±0.04	5.1±0.21
February 2022							
f21	289.2	34.1	20.1	11.1±0.39	129.4±4.22	35.3±1.24	175.8±6.12
f22	399.1	31.7	17.5	0.0±0.0	87.1±3.05	30.8±1.08	117.9±4.13
f23	312.2	35.1	19.5	125.6±4.1	2.1±0.07	1.9±0.07	129.6±3.89
f24	156.3	27.2	17.3	15.5±0.54	11.7±0.41	2.0±0.03	29.1±1.07
f25	178.6	29.1	16.5	56.3±1.97	20.5±0.72	2.6±0.07	79.4±2.89
m21	135.5	27.4	17.1	6.0±0.21	31.1±1.09	2.2±0.02	39.3±1.39
m22	259.2	32.8	12.5	21.5±0.75	37.8±1.32	2.1±0.01	61.4±2.15
m23	176.6	31.5	17.5	9.4±0.33	33.7±1.18	10.7±0.13	53.8±1.88
m24	178.2	28.8	13.0	10.0±0.35	24.3±0.85	3.7±0.02	38.0±2.88
m25	145.5	29.1	12.2	0.6±0.02	31.5±1.10	0.3±0.01	32.4±1.43
March 2022							
f26	305.0	34.5	21.0	11.3±0.09	138.4±4.17	3.2±0.03	152.9±4.43
f27	298.3	31.0	18.8	5.7±0.23	117.0±3.39	8.1±0.17	130.8±3.71
f28	316.2	34.5	19.8	83.4±0.49	193.9±5.62	16.8±2.11	294.2±7.22
f29	308.1	32.7	18.5	15.0±1.63	243.6±7.05	18.2±0.71	276.8±8.89
f30	306.5	32.2	19.0	154.8±1.75	76.1±2.21	19.8±0.87	250.6±2.17
m26	318.6	35.0	19.5	56.3±0.16	91.3±2.65	10.8±0.24	158.4±4.59
m27	276.1	31.0	15.0	60.4±0.17	32.0±0.93	10.8±0.51	103.3±3.19
m28	204.0	33.3	15.0	5.6±0.75	40.4±1.17	3.2±0.06	49.2±1.42
m29	151.2	28.5	15.0	6.0±0.17	52.3±1.52	4.7±0.17	63.1±2.19
m30	256.4	29.6	14.0	60.5±2.78	49.9±1.45	10.9±0.26	121.3±1.89

MU: mouse unit; f: female; m: male

The data collected showed that ten specimens collected in May 2021 were toxic with toxicity varied from 77.1 to 480.6 MU/g. In October 2021, ten specimens accounting for 100% of *C. rotundicauda* specimens were toxic with TTXs toxicity ranging between 57.6 and 156.8 MU/g. For specimens collected in November 2021, five out of ten specimens were determined for toxicity range of 31.1 – 99.4 MU/g, indicating 50% of specimens were toxic. By January 2022, there were 90% non-toxic and 10% toxic specimens identified from ten specimens collected with TTXs toxicity of 21 – 31 MU/g. The specimens collected in February 2022 were assayed and yielded ten out of ten, accounting for 100% of toxic specimens (29.1 – 175.8 MU/g). The collection in March 2022 also resulted in 100% toxic specimens (49.2 – 294.2 MU/g). In sum, the frequency of occurrence of toxic specimens is very high (46 out of 60 specimens; 76.7%). The present data strongly suggest that the frequency of toxic specimens of *C. rotundicauda* in Vietnam is extremely high. This result was in line with the data reported by Dao et al. showing that 83% of *C. rotundicauda* specimens collected in Tan Hai village, Vung Tau province, Vietnam were toxic.[1] The average toxicity determined for all specimens was 96.3 ± 94.2 MU/g, which is considerably higher than 10 MU/g recommended as the safe consumption level of TTX in Japan.[19]

The maximal toxicity of specimens collected in May21, Oct21, Nov21, Jan22, Feb22 and Mar22 were 480.6, 156.8, 99.4, 31.0, 175.8 and 294.2 MU/g, respectively. These toxicity levels were about 2 – 28 fold-time higher than 17.0 MU/g reported for *C. rotundicauda* collected in China by Zheng et al.,[3] 2 – 30 fold-time higher than 16 MU/g reported for Thailand *C. rotundicauda*,[2] and 4 – 65 fold-time higher than 7.4 MU/g determined for Bangladeshi *C. rotundicauda*.[14] However, the toxicity of Vietnamese *C. rotundicauda* collected from Can Gio coast were comparable among the toxicity level reported for Cambodia *C. rotundicauda* (315 MU/g).[13]

It was remarkably noted that 4epi-TTX, TTX and Anh-TTX were three major compounds identified in the specimen's extract (Figure 1) without detection of STXs (Figure 2), making 100% of the total toxicity contributed by TTXs. Consistently, *C. rotundicauda* collected from Cambodia was assayed by LC/MS yielding TTX and its analogues of anhydro-TTX ($[M+H]^+ = 302$) and deooxy-TTX ($[M+H]^+ = 304$) with no PSPs were detected. Contrastingly, Zheng el al. (2019) reported that *C. rotundicauda* collected from China contained TTX, 11-oxoTTX, 4.9-anhydro-11-oxoTTX, 4.9-anhydroTTX, 5-deoxyTTX, 5.11-dideoxyTTX, 5.6.11-trideoxyTTX and 4.9-anhydro-5.6.11- trideoxyTTX in which 5-deoxy TTX in which 4.9-anhydro-11-oxoTTX were found as the major TTX analogues in all specimens. Furthermore, dcGTX2 and dcSTX were also determined with small amount in Chinese *C. rotundicauda* extract.[3] Dao et al. examined *C. rotundicauda* collected from Tan Hai village, Vung Tau province, Vietnam and reported that all specimens contained 4epi-TTX, TTX, Anh-TTX and a certain amount of PSPs (e.g., neoSTX, dcSTX, STX, GTX4, GTX1, GTX3, GTX2, C1, C2) with their composition varied individually.

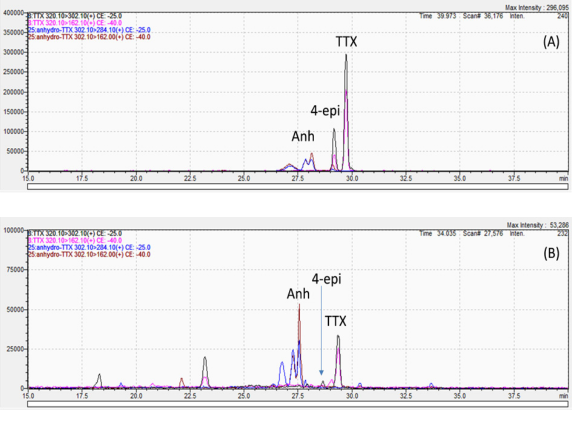

Figure 1. HPLC chromatograms of TTXs standards (A) and in an extract *Carcinoscorpius rotundicauda* specimens (B) collected from Can Gio coast, Ho Chi Minh city, Vietnam.

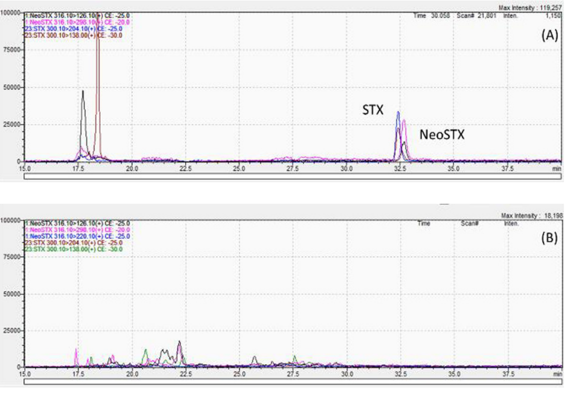

Figure 2. HPLC chromatograms of STXs standards (A) and in an extract *Carcinoscorpius rotundicauda* specimens (B) collected from Can Gio coast, Vietnam.

Statistical analysis of variation of 4epi-TTX, TTX, Anh-TTX and TTXs of *C. rotundicauda* is illustrated in Figure 3A, Figure 3B, Figure 3C and Figure 3D, respectively. Sixty specimens were collected over the study period. For 4epi-TTX, the variation of its toxicity was significant over the months (ANOVA, $F = 2.583$, $p = 0.036$, $n = 10$, Figure 3A). Particularly, the maximal toxicity of 4epi-TTX recorded for ten specimens collected in Mar22 and May21 were 45.9 ± 48.0 and 34.0 ± 34.0 MU/g, respectively, which were significantly higher than those determined of 11.1 ± 14.9 MU/g in Nov21 ($p = 0.039$) and 3.0 ± 4.8 MU/g in Jan22 ($p = 0.013$). Pair comparisons also revealed that 4epi-TTX toxicity determined for specimens collected in Oct21 of 24.2 ± 18.2 MU/g was significantly higher than 3.0 MU/g determined for specimens in Jan22. However, there was no statistically significant difference of 4epi-TTX toxicity of specimens collected in Mar22 and May21 ($p = 0.512$), Mar22 and Feb22 ($p = 0.151$), Mar22 and Oct21 (0.271), May21 and Feb22 ($p = 0.649$), May21 and Oct21 ($p = 0.518$), Feb22 and Oct21 ($p = 0.926$), Feb22 and Nov22 ($p = 0.339$), Feb22 and Jan22 ($p = 0.088$), Oct21 and Nov21 ($p = 0.164$) and Nov21 and Jan22 ($p = 0.164$).

TTX toxicity for specimens collected in May21, Oct21, Nov21, Jan22, Feb22 and Mar22 were 150.1 ± 145, 43.4 ± 6.9, 24.2 ± 28.3, 3.4 ± 3.1, 40.9 ± 38.4, and 103.5 ± 70.5 MU/g, respectively, indicating greater variation among the months (ANOVA, $F = 6.392$, $p = 9.9\times10^{-5}$, n = 10, Figure 3B). Among these pairs, TTX toxicity measured for specimens collected in May21 and Oct21 ($p = 0.044$), May21 and Feb22 ($p = 0.04$), May21 and Nov21 ($p = 0.035$), May21 and Jan22 ($p = 0.010$), Mar22 and Oct22 ($p = 0.023$), Mar22 and Feb22 ($p = 0.039$), Mar22 and Nov21 ($p = 0.014$), Mar22 and Jan22 ($p = 0.002$), Oct21 and Jan22 ($p = 0.000$), Feb22 and Jan22 ($p = 0.015$) and Nov21 and Jan22 ($p = 0.048$) were significantly statistical difference. The remaining pairs including May21 and Mar21 ($p = 0.0266$), Oct21 and Feb22 ($p = 0.826$), Oct21 and Nov21 ($p = 0.080$) and Feb22 and Jan22 ($p = 0.243$) were identified as no statistical difference in TTX toxicity (Figure 3B). Anh-TTX toxicity was also varied significantly over the months (ANOVA, $F = 6.647$, $p = 6.88\times10^{-5}$, $n = 10$, Figure 3C) with maximum and minimum levels of 28.9 ± 15.8 and 1.4 ± 1.3 MU/g determined for specimens collected in Oct21 and Jan22, respectively. The specimens collected in May21, Nov21 Feb22 and Mar22 yielded Anh-TTX toxicity of 13.4 ± 16.9, 4.7 ± 9.3, 9.2 ± 12.9 and 10.7 ± 6.1 MU/g, respectively. Statistical analysis of Anh-TTX toxicity revealed that the pairs e.g., Oct21 and Mar22 ($p = 0.007$), Oct21 and Feb22 ($p = 0.008$), Oct 21 and Nov21 ($p = 0.004$), Oct21 and Jan22 ($p = 0.000$), May21 and Jan22 ($p = 0.043$) and Mar22 and Jan22 ($p = 0.001$) were significantly different. Overall, TTXs toxicity of *C. rotundicauda* collected over the studied months displayed a considerable variation (ANOVA, $F = 10.1$, $p = 7.28\times10^{-7}$, Figure 3D). The mean TTXs toxicity of specimens collected in May21, Oct21, Nov21, Jan21, Feb21 and Mar21 were 197.6 ± 134.5, 96.6 ± 30.6, 39.9 ± 39.5, 7.8 ± 8.4, 75.7 ± 49.7, 160.1 ± 86.4 MU/g, respectively. Typically, measurements of TTXs toxicity in May21 and Oct21 ($p = 0.021$), May21 and Feb22 ($p = 0.026$), May21 and Nov21 ($p = 0.012$), May 21 and Jan22 ($p = 0.001$), Mar22 and Oct 22 ($p = 0.042$), Mar22 and Feb22 ($p = 0.015$), Mar22 and Nov21 ($p = 0.007$), Mar22 and Jan22 ($p = 0.000$), Oct21 and Nov21 ($p = 0.008$), Oct21 and Jan22 ($p = 0.000$), Feb22 and Jan22 ($p = 0.002$) and Nov21 and Jan22 ($p = 0.042$) were exhibited significant differences. Ngy et al.[13] also reported that the toxicity level of TTXs in Cambodia were varied notably between dry (December – January) and rainy seasons (April – May) with generally higher TTXs level observed in rainy months.

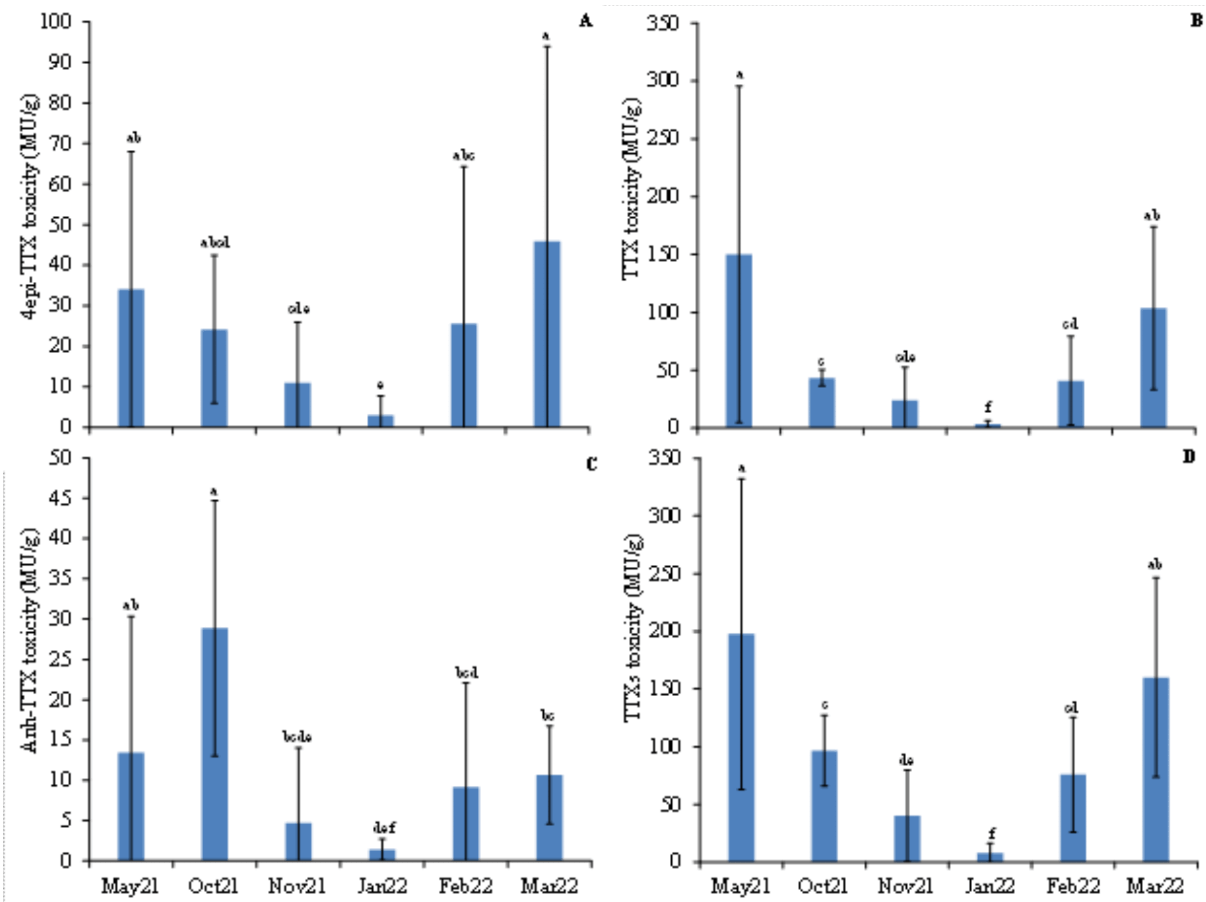

Figure 3. Seasonal variation of 4epi-TTX (A), TTX (B), Anh-TTX (C) and TTXs (D) in *Carcinoscorpius rotundicauda* collected from Can Gio coast, Vietnam in 2021 - 2022. Data is expressed as mean ± SD (n = 10). a, c, e, f are denoted as significant (p < 0.05).

Since horseshoe crab mainly feeds on Mollusca, arthropoda and detritus.[20] Consequently, bacteria or other microorganism inhabiting the decayed organic mattes might be the primary origin of TTX.[21] Therefore, the seasonal variation of TTXs in *C. rotundicauda* can be originated from TTX-bearing organisms which are consumed as their food through several steps of food web,[22] due to seasonal and spatial variation in TTX-producing bacteria community in the aquatic environment.[23] Among these food, TTX-producing bacteria such as *Vibrio* sp. was mentioned elsewhere.[24] On the other hand, toxins in *C. rotundicauda* may originate directly from defense mechanism, in which *C. rotundicauda* use these toxins for their defense to fight with enemy in the environment.[2]

3.2. Tetrodotoxin variation with body weight of C. rotundicauda

In this analysis, total sixty specimens were divided into five different groups. Each group contained 12 specimens with body weight categorized between 100 and 150g (100W150), 150 and 200g (150W200), 200 and 250g (200W250), 250 and 300g (250W300) and heavier than 300g (300W). The data shown in Figure 4 indicates that TTX and its analogues are generally higher levels in *C. rotundicauda* having larger body weight. Particularly, the variation of toxicity of 4epi-TTX (ANOVA, $F = 4.049$, $p = 0.006$, $n = 12$, Figure 4A), TTX (ANOVA, $F = 10.062$, $p = 3.4\times10^{-6}$, n = 12, Figure 4B), Anh-TTX (ANOVA, $F = 4.596$, $p = 0.003$, $n = 12$, Figure 4C) and TTXs (ANOVA, $F = 22.589$, $p = 4.47\times10^{-11}$,

n = 12, Figure 4D) among *C. rotundicauda* groups of 100W150, 150W200, 200W250, 250W300 and 300W are very significant. The maximal 4epi-TTX, TTX, Anh-TTX and TTXs toxicity were determined for 300W groups of 46.0 ± 50.0, 162.4 ± 135.8, 21.9 ± 18.9 and 230.3 ± 116.3 MU/g, respectively, whereas, the minimum levels of 4epi-TTX, TTX, Anh-TTX and TTXs toxicity of 1.7 ± 1.7, 7.3 ± 11.3, 0.8 ± 0.7 and 9.8 ± 12.3 MU/g, respectively, were quantified for 100W150 specimens. Toxicity of 4epi-TTX, TTX, Anh-TTX and TTXs determined for 150W200, 200W250 and 250W300 groups were 14.5 – 33.9 (Figure 4A), 30.1 – 57.7 (Figure 4B), 9.8 – 15.8 (Figure 4C) and 54.3 – 107.5 MU/g (Figure 4D), respectively. This interesting data resonate results obtained from moussed-based experiments conducted by Liao & Li who claimed that adult *C. rotundicauda* was more toxic than young *C. rotundicauda*.[25]

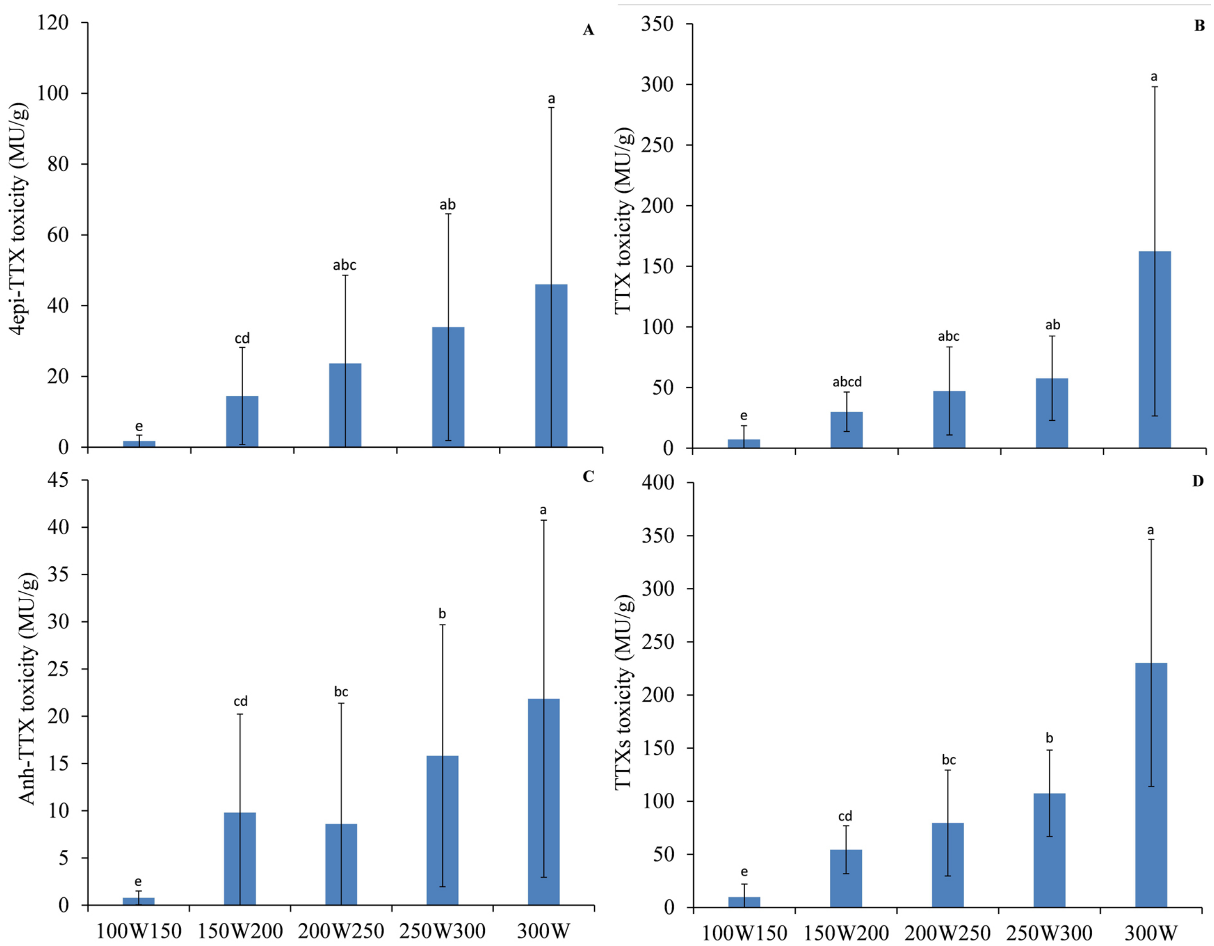

Figure 4. Toxicity variation of 4epi-TTX (A), TTX (B), Anh-TTX (C) and TTXs (D) in five different body-weight groups of *Carcinoscorpius rotundicauda* collected from Can Gio coast, Vietnam in 2021 - 2022. Data is presented as mean ± SD (n = 12). a, b, e are denoted as significant (p < 0.05).

3.3. Tetrodotoxin variation with gender of C. rotundicauda

The total of sixty specimens were divided into two groups of thirty female and thirty male *C. rotundicauda*. The data shown in Figure 5A illustrates that the toxicity levels of 4epi-TTX determined for female and male specimens were 24.6 ± 37.6 and 23.3 ± 26.8 MU/g, respectively, revealing the insignificant difference in variation of 4epi-TTX levels between two *C. rotundicauda* groups (ANOVA, F = 0.025, p = 0.873, n = 30, Figure 5A). Similarity, there was no significant difference of the Anh-TTX levels among female and male *C. rotundicauda* with respective toxicities determined of 14.5 ± 17.6 and 8.3 ± 9.3 MU/g (ANOVA, $F = 2.870$, $p = 0.096$, $n = 30$, Figure 5C). Contrastingly, there was a great variation of toxicity of TTX (ANOVA, $F = 5.298$, $p = 0.025$, $n = 30$, Figure 5B) and TTXs (ANOVA, $F = 5.431$, $p = 0.023$, $n = 30$, Figure 5D) among two groups of *C. rotundicauda.* Basically, female *C. rotundicauda* contained the significantly higher levels of TTX (84.8 ± 110.2 MU/g) and TTX (123.9 ± 121.3 MU/g) than those in male *C. rotundicauda* (TTX, 37.1 ± 26.6 MU/g; TTXs, 68.7 ± 45.8 MU/g). This data is attributed to eggs of female *C. rotundicauda* which generally contain high level of TTXs when compared to those of all soft tissue of male *C. rotundicauda*.[3,13,14]

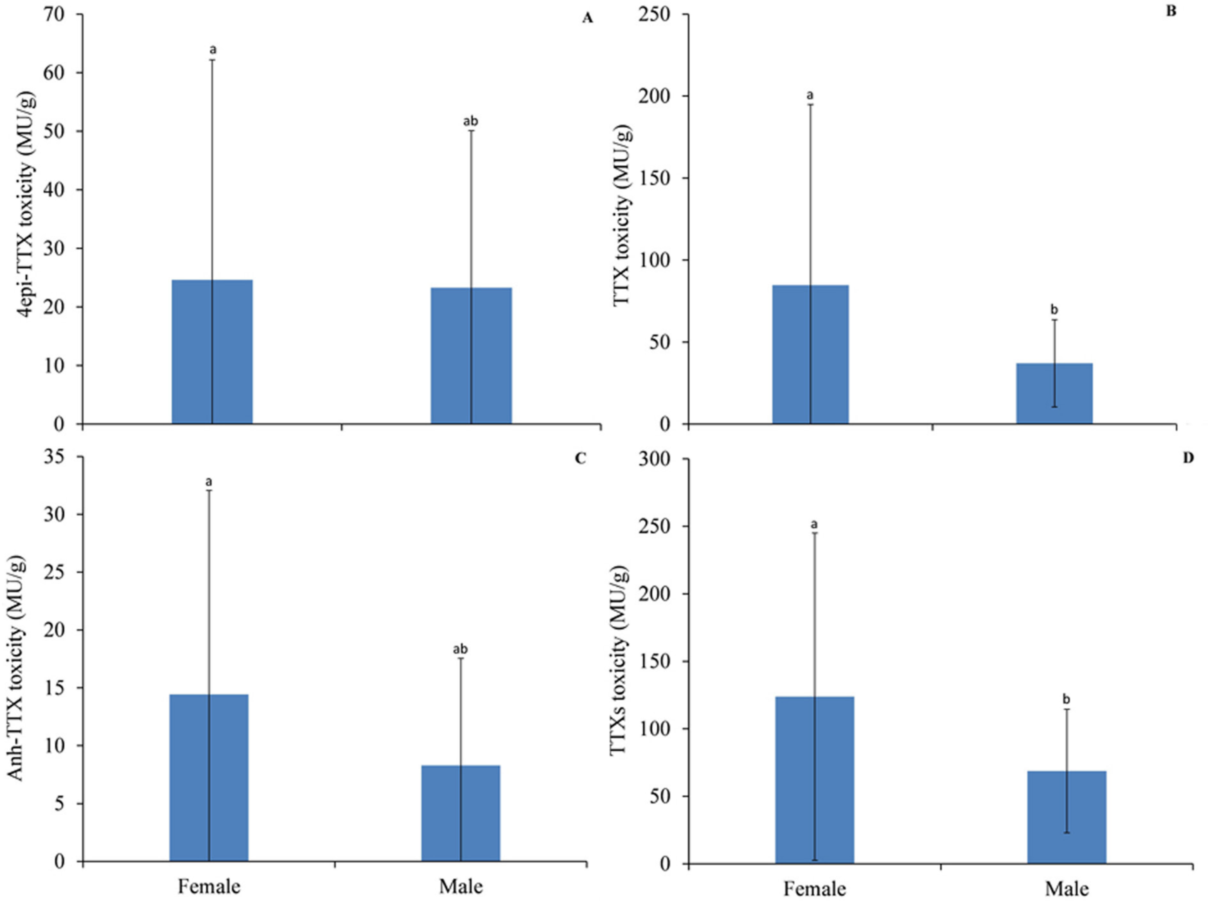

Figure 5. Toxicity variation of 4epi-TTX (A), TTX (B), Anh-TTX (C) and TTXs (D) in two different *Carcinoscorpius rotundicauda* female and male groups collected from Can Gio coast, Vietnam in 2021 - 2022. Data is presented as mean ± SD (n = 30), a, b are denoted as significant ($p < 0.05$).

Overall, toxicity and toxin composition of *C. rotundicauda* are principally varied depending on regional and individual variations.[1,3,13,14] Moreover, results obtained in this study further demonstrated that toxicity and toxin composition of *C. rotundicauda* are also season, gender as well as age variation. Despite considerable variation in TTXs composition and toxicity in Vietnamese *C. rotundicauda* over the months, the majority of specimens were toxic with the mean TTXs toxicity level of 96.3 ± 94.2 MU/g, suggesting that *C. rotundicauda* collected from Vietnam is not suitable for human consumption. Nevertheless, this is a promising source for isolation and purification of TTXs for research and development applications.

4. CONCLUSIONS

TTXs contributed 100% toxicity of *C. rotundicauda* collected from Can Gio coast, Ho Chi Minh City, Vietnam in May 2021 to March 2022. There were significant variations of TTXs level over the months with maximal TTXs toxicity determined of 197.6 ± 134.5 MU/g for specimens collected in May 2021, whereas the *C. rotundicauda* collected in January 2021 was found to yield minimum TTXs level of 7.8 ± 8.4 MU/g. Interestingly, the difference of TTXs level in different body-weight *C. rotundicauda* groups was also statistically significant, with maximal TTXs toxicity of 230.3 ± 116.3 MU/g determined for specimens having body weight of greater than 300g, while the lowest level of TTXs (9.8 ± 12.3 MU/g) was assayed for *C. rotundicauda* group of body weight less than 150g. Moreover, there was a significant difference in TTXs levels among female and male *C. rotundicauda* groups, with notable higher TTXs toxicity determined for female specimens (123.9 ± 45.8 MU/g) than that of male *C. rotundicauda* (68.7 ± 45.8 MU/g). The data obtained in this study further confirmed that this is a promising source for isolation and purification of TTXs for research and development applications.

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