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The first report on the marine snail *Phos senticosus* (Linnaeus, 1758) causing a tetrodotoxin poisoning in Viet Nam

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Abstract

The neurotoxic poisonings that result in death with fatalities caused by consumption of *Nassarius* snails have been reported in Viet Nam but the causative toxin has only been identified in a small number of cases. A poisoning incident by eating marine snails happened in Binh Thuan Province, Viet Nam in March 2021. The leftover food from the incident, 29 snail specimens, later were identified as all *Phos senticosus* (Linnaeus, 1758) were collected for identification of causative toxin. At 60.7 ± 126.5 μg/g, anh-TTX was detected in the most dominant level, followed by tetrodotoxin (TTX) (46.0 ± 107.6 μg/g) and 4-epiTTX (23.3 ± 24.3 μg/g) by hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) analysis. Overall toxicity was assessed to be 229 ± 526 MU/g in the snail specimens that responded mostly to TTX due to its strong potency. All specimens were reported as toxic in toxicity range of 10–2,672 MU/g, which is comparable to what was found in some other *Nassarius* species in Taiwan and Viet Nam. Of 29 specimens, 75.8% showed toxicity in the range of 10–100 MU/g, 17.3% indicated toxicity in the range of 100––1,000 MU/g, whereas 6.9% displayed toxicity that was exceptionally high (> 1,000 MU/g). The data demonstrates that TTX is accountable for this poisoning incident. Notebly, it is the first report on TTX in *P. senticosus* causing the seafood poisoning in Viet Nam, highlighting the threat this species poses to human consumption. As a result, there ought to be a more severe warning about its potential cause of poisoning.

Keywords: HILIC-MS/MS, Poisoning, *Phos senticosus*, Tetrodotoxin, Viet Nam

Introduction

Marine snails are popular seafood in Asian countries, and a certain number of poisoning cases have been reported due to eating this animal group (Ha & Sato, 2010; Ha et al., 2023; Hwang et al., 1995, 2005; Shiu et al., 2003; Shui et al., 2003; Sui et al.,

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2002; Yang et al., 1995). Several marine snail's families were known as causative organisms for these poisonings (Noguchi et al., 2011). It was indicated that tetrodotoxin (TTX) and saxitoxins are responsible toxins in these poisonings (Ha et al., 2020; Hwang et al., 2004, 2007; Liu et al., 2004; Narita et al., 1984; Taniyama et al., 2009, 2013).

TTX, a powerful sodium channel blocker of excitable membranes, is one of the marine toxins linked to human poisonings. The origin of TTX contamination in marine environments is still under investigation, with various hypotheses proposed (Varini et al., 2025). It has been known to spread widely, but only in a few and specific species of freshwater and marine cold-blooded organisms (Hwang et al., 1992; Kim et al., 1975; Lin & Hwang, 2001; Miyazawa & Noguchi, 2001; Mosher & Fuhrman, 1984), bacteria (Noguchi et al., 1986; Yasumoto et al., 1986) and macroalgae (Yasumoto et al., 1988). TTX poisoning has been reported mainly in Southeast Asia, China, Korea, Japan, where the potential toxic animals such as puffer fish, horshoe crab, toxic goby, toxic snails and others are eaten (Hashimoto, 1979; Mosher & Fuhrman, 1984).

In Viet Nam, poisoning incidents by eating marine snails have been happened, sometimes, mostly in coastal areas (Ha & Sato, 2010; Ha et al., 2020). In our preminary studies, 05 marine Nassaridae snail species (*Nassarius conoidalis*, *Nassarius glans*, *Nassarius papillosus*, *Nassarius pullus*, and *Nassarius siquijorensis*) were found to contain TTXs (Dang et al., 2015; Ha et al., 2020). This data indicated the potential threat to human health from marine snails in Viet Nam. However, in almost of all case, causative toxin(s) in poisoning incidents have not been confirmed due to lacking of specimen collection, excepted in the incident caused by eating *Nassarius glans* in Khanh Hoa 2020 (Ha et al., 2020).

In March 2021, there was a poisoning incident involving 05 family members as result of eating marine snails in Phan Thiet City, Binh Thuan Province, Viet Nam. About 30 min after eating, four out of five persons were experienced with typical neurological symptoms such as tingling on lips, touge and limbs. This paper presents result of toxin analysis in the implicated snail samples, which was later identified as *Phos senticosus* (Linnaeus, 1758) (Fig. 1) collected in the incident. Also, the toxicity variation of 29 collected snail individuals was documented. It is the first scientific report on *P. senticosus* causing poisoning in Viet Nam, therefore, it is important for public awareness on human health risk from this marine species.

Fig. 1. Photo of *Phos senticosus* (Linnaeus, 1758) specimen collected from the poisoning incident in Binh Thuan, Viet Nam in March 2021.

Materials and Methods

Specimen collection

As leftover food, 29 snail individuals were collected in the poisoning incident in Phan Thiet City, Binh Thuan Provice in March 2021 and sent to the laboratory in cool condition. Each snail specimen was cleaned outside, identified scientific name, measured and deshelled to collect soft tissue (Table 1) for toxin analysis.

Chemicals

Formic acid, acetic acid and TTX (1 mg) were Wako pure chemicals (FUJIFILM, Osaka, Japan) products. Ammonium hydroxide of 25% (liquid chromatography-mass spectrometry [LC-MS] grade) was a Sigma-Aldrich (Tokyo, Japan) product. Acetonitrile was Kanto Chemicals (Tokyo, Japan) product. 4-epiTTX and anh-TTX were a gift from Dr. Shigeru Sato, Kitasato University, Japan.

Analysis of toxins

For food safety consideration, all snail soft tissue that is frequently consumed by people was used for toxin analysis. Additionally, the soft tissue of each individual snail was only around

Table 1. Information on marine snails collected from the poisoning incident in Binh Thuan, Viet Nam in 2021

n	Length (cm)	Width (cm)	Whole body weight (g)	Weight of soft tissue (g)
29	3.86 ± 0.29	1.98 ± 0.18	5.79 ± 1.03	2.70 ± 0.63

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2 g (Table 1) due to the snail species' small size, replicates were not applied during the extraction procedure. Since TTXs was the predominant toxin in a number of marine toxic species in Viet Nam, this toxin was the focus of this investigation. TTXs in the soft tissue was extracted following to Brillantes et al. (2003). Briefly, the soft tissue was homogenized with 1% acetic acid (1:4 w/v), boiled for 5 min, cooled down at room temperature and centrifuged at 11,000×g, 25℃ for 10 min to collect the extract, which one ml was equivalent to 0.2 g of the soft tissue. The extract was then passed through an ENVI-Carb SPE cartridge (250 mg, 3 mL^{-1}, Sigma-Aldrich, St. Louis, MO, USA), eluted by acetonitrile 25%. TTXs in the eluates were then dertemined by the hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) method following to Boundy et al. (2015) with some modifications using the liquid chromatography (LCMS 8040, Shimadzu, Kyoto, Japan) coupled to a triple quadrupole/linear ion trap mass spectrometer DUIS-8040 (Shimadzu). The HILIC separation was carried out on a Waters Xbrige (HILIC) Amide column (4.6 mm I.D. × 150 mm, 3.5 μm, WatersTM, Milford, MA, USA) at 60℃ with injected volume of 5 μL. Water/formic acid/ammonium hydroxide (500:0.075:0.3 v/v/v) (A) and acetonitrile/water/formic acid (700:300:0.1 v/v/v) (B) were set in a flow rate of 0.6 mL min^{-1} as mobiphases. The chromatographic conditions were initial condition of 100% B for 20 min; following of a linear gradient of 50:50 A and B within 15 min, held for 9.90 min.

The ion source parameters of the MS spectrometer were 10 V of entrance potential, 30 psi of curtain gas; 4,500 V of ion spray voltage; 250℃ of source desolvation temperature; 400℃ of source ion block temperature; 1,000 Lh^{-1} of desolvation gas flow; 2 L min^{-1} of nebulizer gas flow; and 0.15 mL min^{-1} of collision gas flow rate. Multiple reaction monitoring (MRM) was performed in a positive electrospray ionization. Due to the limitation of reference material (TTX standard), single-point calibration was applied in the analysis, also recovery test with spiked samples were not carried out in this study. Practically, the limit of detection and the limit of quantitation were 38 nM and 126 nM for TTX analysis on our HILIC-MS/MS system. MS/MS spectra were obtained with 40 eV of collision energy for the precursor of *m/z* 320.0 within a range of *m/z* 50–350 for confirmation of TTX in the snail extract.

TTX levels were calculated from HILIC-MS/MS data by comparing with TTXs standard. Toxicity was expressed in mouse unit (MU/g) in which one MU is the dose of toxin that will kill a male mouse (ddY, 20 ± 2 g body weight) within 30 min. One mg TTX is corresponding to 4,500 MU, one mg 4-epiTTX to 709 MU and one mg anh-TTX to 92 MU of toxicity (Nakamura & Yasumoto, 1985).

Results

The HILIC/MRM chromatogram revealed that the retention times (Rt) for the anh-TTX, 4-epiTTX and TTX standards were 24.17, 25.58 and 26.50 minutes, respectively (Fig. 2). The corresponding peaks that were seen in all extracts from soft tissue of *P. senticosus* indicating the presence of TTXs. The MS/MS spectrum at *m/z* 162, 178, 256, 284, 302 and 320; which match to the indentical MS/MS spectrum of the TTX standard were detected

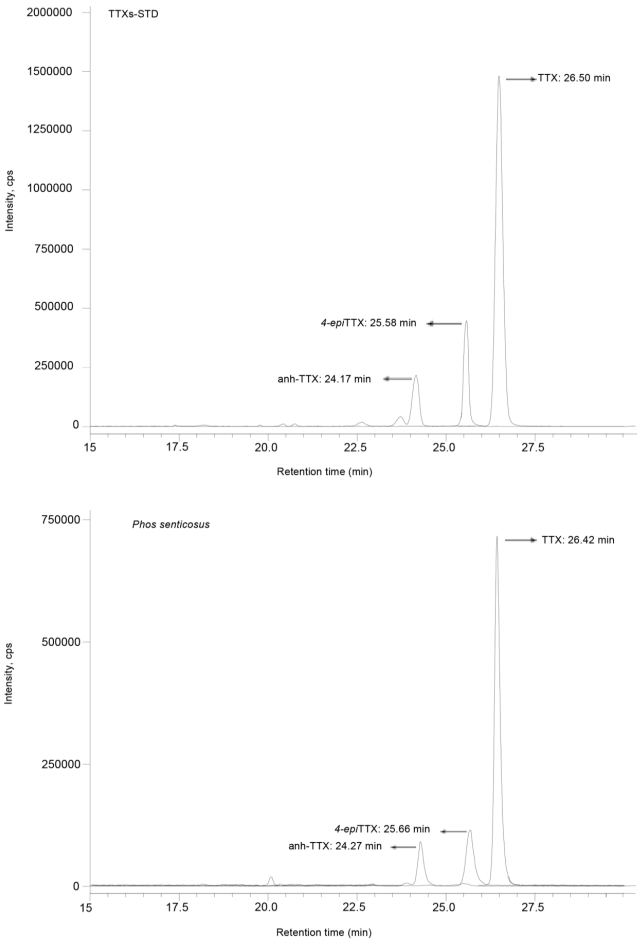

Fig. 2. Hydrophilic interaction liquid chromatography/multiple reaction monitoring (HILIC/MRM) chromatography of tetrodotoxins (TTXs) standard (anh-TTX: 0.35 μg mL^{-1}; 4-epi TTX: 0.43 μg mL^{1}; TTX: 0.5 μg mL^{-1}) and in the extract of *Phos senticosus* specimen.

in the snail extract, confirmed TTX in the sample (Fig. 3).

Table 2 presents level of TTXs and overall toxicities (MU/g) calculated based on the specific mouse toxicity of each toxin component (Nakamura & Yasumoto, 1985) in 29 specimens of *P. senticosus*. At 60.7 ± 126.5 μg g^{-1}, anh-TTX was detected in the most dominant level, followed by TTX (46.0 ± 107.6 μg g^{-1}) and then 4-epiTTX (23.3 ± 24.3 μg g^{-1}). The overall toxicity in these specimens was estimated as 229 ± 526 MU g^{-1} with the range from 10 to 2,672 MU g^{-1}, with 90% was responsible from TTX due to it strong potency.

All specimens were recognized as toxic, with 75.8% exhibiting toxicity in range of 10–100 MU g^{-1}, as shown in Table 3. In particular, 17.3% specimens had TTX toxicity between in range 100–1,000 MU g^{-1}, whereas 6.9% displayed toxicity that was exceptionally high (> 1,000 MU g^{-1}) (Table 3).

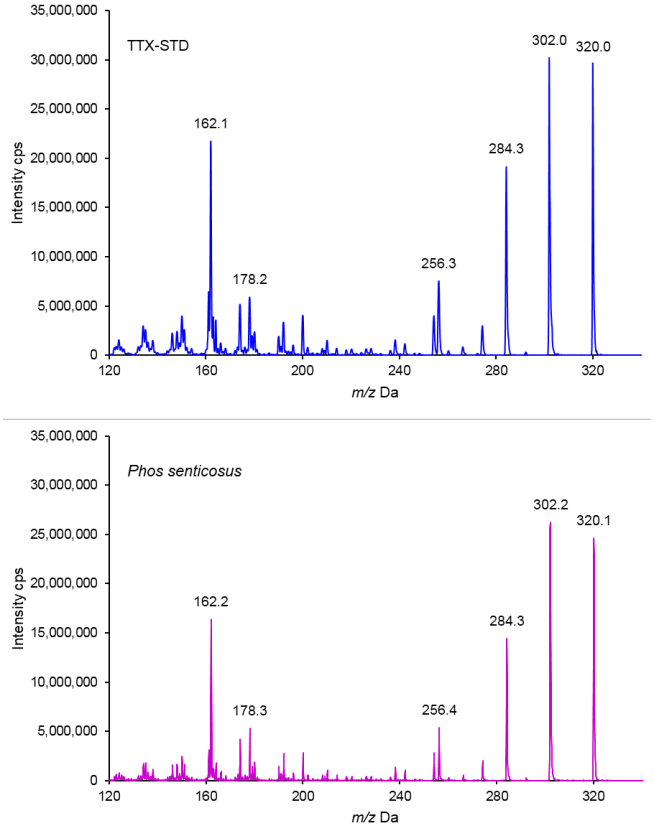

Fig. 3. Tandem mass spectrometry (MS/MS) spectra of tetrodotoxin (TTX) standard at 0.5 μg/mL and TTX in the soft tissue extract of *Phos senticosus* (a collision energy of 40 eV for the precusor of *m/z* 320.0).

Table 2. Level of tetrodotoxins (TTXs) and overall toxicity in Phos senticosus specimens (n = 29) collected from the poisoning incident in Binh Thuan, Viet Nam in March 2021

Value	Level of TTXs (μg/g)			Overall toxicity (MU*/g)
	anh-TTX	4-epiTTX	TTX	
Range	5.3–603.9	1.9–233.7	1.7–544.5	10–2672
Mean ± SD	60.7 ± 126.5	23.3 ± 24.3	46.0 ± 107.6	229 ± 526

*One MU is the dose of TTXs which kills a 20 g male mouse (ddY) in 30 min (Nakamura & Yasumoto, 1985).

Discussion

The amount of TTX found in *P. senticosus* specimens was less than the amount of anh-TTX, as shown in Table 2. However, TTX was responsible for most of the total toxicity because its toxic potency (4,500 MU mg^{-1}) was over 50 times higher than that of anh-TTX (92 MU mg^{-1}) which noted by Nakamura and Yasumoto (Nakamura & Yasumoto, 1985). It shows that the toxin that caused this poisoning incidence was TTX.

Among more than 15 *Nassarius* snail species known to be distributed in Viet Nam (Hylleberg & Kilburn, 2003), 5 species were reported to contain certain levels of TTX (Dang et al., 2015; Ha et al., 2020) and later, one of them, *Nassarius glans* was confirmed to cause the poisoning incident in Khanh Hoa Province, 2020 (Ha et al., 2023). *P. senticosus* (Linnaeus, 1758), which is also member of Nassariidae family, known to distribute widly in Indo-West Pacific (Abbott, 1991; Cernohorsky, 1972; Okutani, 2000; Wilson, 1994); however, there is little information available regarding TTX this species. The present data is the first report on the presence of TTX in *P. senticosus*, also it supports our previous data that TTX is a dominant toxin in marine snails in Viet Nam (Ha et al., 2020). According to the findings, it is suggested that *Nassarius* snails pose a health concern to people in Viet Nam.

The toxicity level detected in the specimens was all beyond the safe level of consumption (10 MU/g) for puffer (fish) suggested in Japan (Kodama & Sato, 2005) and the European Food Safety Authority (EFSA) for TTX (which sets a safety limit of 44 μg/kg TTXs in shellfish meat) (EFSA et al., 2017). The highest TTX toxicity in this study was higher than that detected in

Table 3. Frequency (%) of tetrodotoxin (TTX) toxicity in range of *Phos senticosus* specimens (n = 29) collected from the poisoning incident in Binh Thuan, Viet Nam in March 2021

TTX toxicity range (MU/g)	10–100	100–1,000	> 1,000
Number of specimens	22	5	2
Frequency (%)	75.8	17.3	6.9

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some other *Nassarius* species in Viet Nam in our earlier report (Ha et al., 2020) and comparable with that detected in *Nassarius glans* in Taiwan (Hwang et al., 2005). It is claimed that *P. senticosus* in Viet Nam is unsafe for human consumption, even if this level is still lower than that of *Nassarius glans* from Viet Nam in our latest report (Ha et al., 2023) and Japan (4,290 MU g^{-1}) (Taniyama et al., 2009). The minimum human lethal dose of TTX is estimated to be approximately 10,000 MU (Noguchi et al., 2011). Similar to many other marine snails, *P. senticosus* is frequently eaten by not only locals but also foreign tourists in Viet Nam. According to the present data, only 10 g soft tissue (equivalent to 4 specimens) containing maximum toxicity (2,672 MU g^{-1}) may cause death for people if consumed. In addition to that, frequency of toxic specimens of *P. senticosus* in this study was quite high (100%) with a certain number of extremely toxic specimens (6.9%). Although the number of specimens in this study was small, the results indicate that *P. senticosus* at least in Viet Nam is unsafe, even quite dangerous for human food. More attention has to be paid to this possible source of poisoning.

Certain snail species, particularly marine snails like the trumpet shell, can accumulate high concentrations of TTX (Costa et al., 2021). This accumulation can occur through the food chain, where snails ingest TTX-bearing organisms like certain starfish or dead puffers, leading to trophic transfer of the toxin (Noguchi & Arakawa, 2008). There was a hypothesis that the origin of TTX in the small necrophagous snails including Nassariidae species originate from their food, however still uncertain due to geographical dispersion and seasonal variation (Noguchi et al., 2011). For instance, in contrast to spring to early summer in China and Taiwan or late summer in Japan (Noguchi et al., 2011), snail poisoning incidents were observed in Viet Nam in autumn (Ha et al., 2023). TTX in *P. senticosus* together with their food sources would be an interesting for understanding the mechanism of toxin contamination in this marine animal's group.

Conclusion

For the first time, TTX was verified as the responsible toxin in the marine snail *P. senticosus* specimens causing the poisoning incident in Binh Thuan Province, Viet Nam in March 2021 by HILIC-MS/MS analysis. Given the high level and wide range of TTX toxicity found in the specimens, it is unsuitable, even dangerous, for human consumption, at least in Viet Nam. There should be a more stringent warning about this species' potential to cause poisoning. To further understand the genesis of the toxin and its accumulated mechanism, more research is required on the geographical and seasonal variation of TTX in *P. senticosus*.

Competing interests

No potential conflict of interest relevant to this article was reported.

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Availability of data and materials

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Ethics approval and consent to participate

This study confirmed to the guidance of animal ethical treatment for the care and use of experimental animals.

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