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Assessment of proximate composition and tetrodotoxin content in the muscle of Yellow puffer fish, *Xenopterus naritus* (Richardson 1848) from Sarawak, Malaysia

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Abstract

This study is to report the proximate compositions as well as tetrodotoxin (TTX) content in the muscles of yellow puffer fish *Xenopterus naritus* that collected from Kg. Manggut and Kabong, Sarawak. The internal organs of 26 and 20 specimens from Kg. Manggut and Kabong respectively were removed by the local people that had skills and experiences with the preparation of yellow puffer fish. In general, the moisture contents were ranging between 75.2% and 80.6%. *X. naritus* from Kabong showed higher crude protein contents (88.2% dry weight) than the same species from Kg. Manggut (87.9% dry weight) and not significantly different ($p>0.05$). *X. naritus* from Kg. Manggut demonstrated a significantly higher ($p<0.05$) of crude fat contents (0.49% dry weight), crude fibre contents (0.44% dry weight) and ash contents (5.48% dry weight) compared to *X. naritus* from Kabong which were 0.47%, 0.25% and 5.08% dry weight respectively. The TTX content in the muscle of *X. naritus* that prepared by the unskilled person from Kg. Manggut showed significantly higher (65.3 µg/g) ($p<0.05$) than *X. naritus* that prepared by the skilled person from Kabong (6.63 µg/g) and found to be toxic for human consumption (>2 µg/g) based on Japanese regulation as Malaysia does not have the regulatory limits for TTX yet. Nevertheless, it can be consumed safely if prepared in a proper manner. This is the first report to determine the proximate composition from the muscle of yellow puffer fish from Sarawak. The proximate values obtained from this study shows that the yellow puffer fish *X. naritus* are good protein resources. Therefore, it can be considered for human consumption in countries. The information gained from this study indicates the importance removing TTX to ensure the safe consumption of puffer fish.

Introduction

The puffer fish, *Xenopterus naritus* (Richardson, 1848), or Yellow puffer fish (Figure 1) belongs to the family Tetraodontidae. It can be easily identified by the prominent yellowish or golden coloration especially at the lower part of the body and exhibit a torpedo-shaped body. This puffer fish is a migratory species that inhabits the South China Sea and return to the river to spawn. The juvenile inhabit coastal water out to the sea during the non spawning season. It is widely distributed in China, Thailand, Vietnam, Indonesia and Malaysia. According to Gambang and Lim (2004), in Malaysia, yellow puffer fish is abundant only in Sarawak and the fish is found in the coastal waters especially in areas fringing the mangroves particularly along the Batang (River) Saribas in Betong, Sarawak. It is famous amongst the local people and locally known as 'ikan buntal kuning'.

Figure 1. Yellow puffer fish *Xenopterus naritus*

In Malaysia, puffer fishes are easily found and classified as trash fish. They have no market value and not consumed by local people but in Sarawak, yellow puffer fish is considered a delicacy by the local community particularly in Manggut Village area. Due to its good taste, various dishes and products of this fish is processed and prepared. In Sarawak, celebration of the 'Yellow puffer fish Festival' has become a tourist attraction every year in August at

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Manggut Village, Betong, Sarawak.

Puffer fish belong to Tetraodontidae family is probably the most common fish that known to possess a neurotoxin or tetrodotoxin (TTX) which can cause a puffer fish poisoning. Although the locals are aware of the poisonous effect of yellow puffer fish, this fish and its products are easily available and much sought after by the locals. The price of dried salted puffer fish eggs in Sarawak can reach up to RM 30-40 per kilogram (USD 10-14) (Muliadi and Muhammad Raduan, 2008). The presence of toxin in this fish and its products could be more harmful because they are eaten whole as the toxin could not be removed easily. Despite the use of puffer fish as a food is rare, there was a record of food poisoning cases in Malaysia. The toxicity of yellow puffer fish *(X. naritus)* has been documented from Andaman Sea (Kungsuwan, 1994), Sg Saribas, Sarawak (Bojo *et al*., 2006; Mohamad *et al.*, 2008) and Batang Sadong, Sarawak (Che Nin *et al*., 2010). Most of these cases were caused by ingestion of contaminated puffer fish species. TTX intoxications mostly occur in Japan and other Asian countries, where puffer fish is served as a delicacy.

Although yellow puffer fish have commercial value for the local people in Sarawak, they do not pay attention on the nutritional values of this species. At present, there is no information on nutritional values of yellow puffer fish. Thus, this study was attempted to determine the nutritional content of the edible part of yellow puffer fish. Generally, it is consider that fishes from different types contain the same nutritional value (Osman *et al*., 2001). Due to little published information is available on the toxicity of the edible part of yellow puffer fish, this study is also to determine the TTX concentration from the muscle of yellow puffer fish caught from Sarawak.

Materials and Methods

Specimen collection

A total of 56 and 38 specimens of the yellow puffer fish *X. naritus* were caught using trammel net or gill net by local fishermen from Kg. Manggut (1°31'55.90"N, 111°20'9.60"E) and Kabong (1°49'18.2"N, 111°07'51.5"E) (Figure 2), Sarawak respectively between February and July 2013. The specimens were kept in plastic thermal insulated boxes with ice within 4 – 5 hrs and transported to the laboratory of the Fisheries Research Institute Sarawak, Bintawa, Sarawak, and subsequently kept frozen.

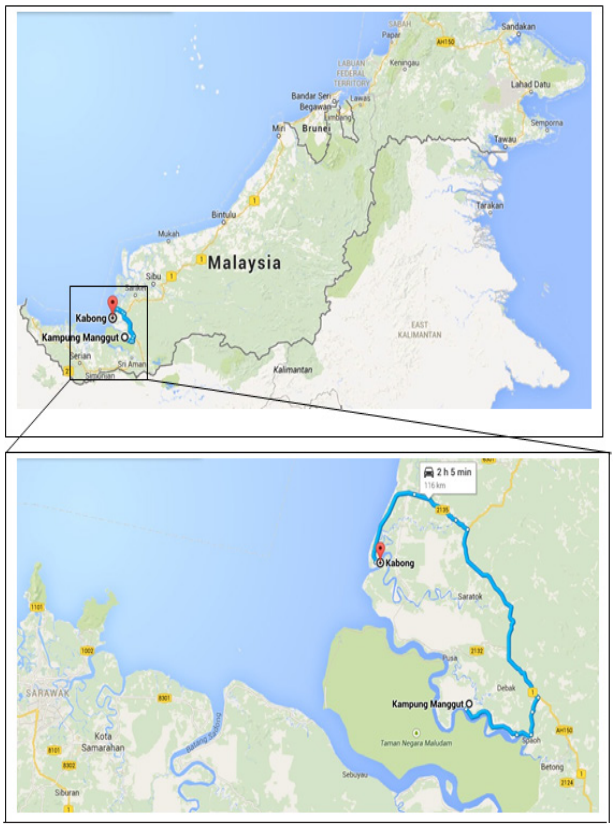

Figure 2. Location of yellow puffer fish *X. naritus* obtained in the present study

Sample preparation

The specimens were identified based on the morphological characteristics (FRI, 2004; Froese and Pauly, 2011). Then, the specimens were individually measured for their total body weight and length. In this study, the internal organs of yellow puffer fish were removed by skilled and unskilled person. The internal organs of 26 and 20 specimens from Kg. Manggut and Kabong respectively were removed by the fisherman that had skills and experiences with the preparation of yellow puffer fish. The belly cavity of the fish that contain the entrails and viscera was removed completely and thoroughly by cutting along the belly to end without leaving traces. The bile of the eggs should be handled with care and must be completely removed to ensure the eggs are safe to eat (Parvaneh *et al.,* 2012). Meanwhile, the other specimens were dissected by the unskilled person. The skin of each specimen was removed and the fish fillets were separated from both sides with a knife and weigh. Then the filleted specimens were minced and used for proximate and tetrodotoxin content analysis separately. Eighteen specimens from each location were used for proximate analysis. In this study, all specimens were used to determine the TTX content. Each minced tissue was packed in zip lock plastic bag (4½" x 6"), labeled accordingly and kept at -20°C until delivered to Fisheries Research Institute, Penang

for proximate and tetrodotoxin content analysis.

Proximate analysis

Moisture content analysis

Moisture content of yellow puffer fish minced fillets was determined according to the method described by AOAC (2000) with slight modifications. The samples (1 g) were weighed (MS304S Mettler Toledo, Switzerland) and dried in crucible in an oven (UFB400 Memmert, Germany) at 105˚C until constant weights were obtained. Meanwhile, about 100 g of the minced fillets were also dried in moisture dish in an oven as described previously for moisture content analysis. After constant weight was obtained, the samples were ground using a dry grinder (MX-800S Panasonic, Malaysia) and fine powders that obtained were used for ash, crude protein, crude fat and crude fibre content analysis.

Ash content analysis

Ash content of yellow puffer fish minced fillets was determined according to the method described by AOAC (2000) with slight modifications. Dried samples from the moisture content analysis were ashed in a muffle furnace (Carbolite, UK) at 550˚C overnight.

Crude protein analysis

Crude protein content of yellow puffer fish minced fillets was determined according to the method described by AOAC (2000) with slight modifications as recommended by Kjeltec 2100 (Foss Analytical, Denmark). Briefly, 1 g dried sample was weighed into digestion tubes. Two Kjeltabs Cu 3.5 (catalyst salts) were added into each tube. About 12 ml of concentrated sulphuric acid (H_2SO_4) was carefully added into the tube and then shaken gently. Digestion procedure was performed using pre-heated (420˚C) digestion block of 2066 Digestor (Foss Tecator, Sweden) for 60 min until clear blue/green solution was obtained. Digested samples were cooled for 20 min. Distillation procedure was then performed using distillation unit of 2100 (Foss Tecator, Sweden). Distillate was titrated using 776 Dosimat (Metrohm, Switzerland) with 0.5N hydrochloric acid (HCl) until pinkish end point achieved.

Crude fat content analysis

Crude fat was extracted from the dried sample (1 g) following the method described by AOAC (2000) using the Soxtec 2055 System (Foss, Sweden) with petroleum ether as the solvent. The content of crude fat was determined gravimetrically after oven drying (105˚C) the extract overnight.

Crude fibre content analysis

Crude fibre was determined by hot extractor Fibertec 2010 (Foss Tecator, Sweden) with sequential extraction of dried samples (1 g) with 1.25% H_2SO_4 (1 L) and 1.25% NaOH (1 L) using the Borosilicate 3.3 (Foss, Sweden) as a container. For drying and ashing, the crucible with sample was dried in an oven for 5 hrs at 105˚C and ashed in the muffle furnace (Carbolite, UK) at 550˚C overnight. The weight of crucible with sample after drying and ashing was recorded and the crude fibre content was calculated (AOAC, 2000).

Sample extraction for toxin analysis

The minced fillets were determined in duplicate according to the Japanese Official Method (Japan Food Hygiene Association, 2005) with slight modifications. A small portion (2 g) of minced fillets was subjected to an equal volume of 0.1% acetic acid (AcOH) in 15 ml centrifuge tube. The samples were then homogenized using an ultrasonic probe (OMNI-Ruptor 4000, Georgia, USA) for 1-3 min. The mixture was heated in a boiling water bath (BUCHI B-480, Germany) for 10 min and then cooled in slurry ice and centrifuged at 5,000 rpm for 15 min (Eppendorf 5430, Hamburg, Germany). The supernatant obtained was filtered through a 0.45 µm nylon membrane filter before analysed using liquid chromatography-mass spectrometry mass spectrometry (LC-MS/MS).

Analyses by LC-MS/MS

Mass spectrometric detection was performed with a TSQ Quantum Discovery MAX (Thermo Electron, USA) consisting of an MS Surveyor pump with autosampler coupled to a mass spectrometer equipped with an electrospray ionisation (ESI) probe. The mass spectrometer was operated in positive ESI. Optimal ion source and interface conditions were achieved at a spray voltage of 3,500V, sheath gas flow of 12 units, auxiliary gas flow of 3 units, collision energy (CE) of 18, collision gas pressure of 1.5 mTorr and capillary temperature of 300˚C. Peak detection, data acquisition and the calibration graph plot were performed using the Xcalibur 2.1.0 software. A ZIC-HILIC column (SeQuant, Haltern, Germany) (150 mm x 2.1 mm, 5 µm) with a guard column (20 mm x 2.1 mm, 5 µm) (SeQuant, Haltern, Germany) was used to separate TTX (Diener *et al*., 2007; Mohd Nor Azman *et al.*, 2013). Mobile phase A was 10 mM ammonium formate (NH_4COOH) and 10 mM formic acid (CH_2O_2) in water and mobile phase B was 5 mM NH_4COOH and 2 mM CH_2O_2 in acetonitrile/water

(ACN/H_2O) (80:20, v/v). A gradient elution was used at a flow rate of 250 µl/min. The gradient programme was applied as described by Diener *et al.* (2007). The gradient started at 100% B, which was decreased linearly to 65% B in 0.1 min. It was kept at 65% B for 7.0 min and returned in 3.0 min to 100% B. An equilibration time of 20 min was allowed before the next injection.

Statistical analysis

Data were analysed by the statistical software of SPSS (Statistical Package for the Social Sciences) version 16.0 for Windows. The data were transformed to a normal distribution prior to analysis. One way analysis of variance (ANOVA) was used to compare differences in the means of the moisture content, ash content, crude protein content, crude fat content and crude fibre content of puffer fish. This was followed by Duncan multiple range test analysis to determine the differences between samples. T-test was applied to the toxicity data to compare differences in the mean of TTX of different locations of puffer fish. All proximate compositions were analysed in triplicate and reported as mean on % dry weight basis. Means±SD of triplicate determinations were considered significantly different when $p<0.05$.

Results

Length and weight comparison

A total of 56 and 38 individuals of *X. naritus* were collected from Kg. Manggut and Kabong respectively between February and July 2013. The mean total length of Kg. Manggut specimens and Kabong specimens was 18.1±3.93 cm and 23.2±2.26 cm respectively and significantly different ($p<0.05$). The mean total length and body weight of Kabong's specimens were significantly higher than Kg. Manggut's specimens ($p<0.05$).

Proximate composition

The proximate composition of the muscle of *X. naritus* from Kg. Manggut and Kabong, Sarawak including the moisture, crude protein, crude fat, crude fibre and ash contents are presented in Table 1. The moisture content of the muscle samples in general was found to be between 75.23% and 80.63%. The mean moisture contents of *X. naritus* from Kabong, Sarawak were measured as 79.97±0.56% and significantly higher than *X. naritus* collected from Kg. Manggut, Sarawak which measured as 77.36±1.13% ($p<0.05$).

The crude protein content was detected to be relatively high and ranged between 84.4% and 92.8% (dry weight basis), which was similar and not significantly different between the samples collected from Kabong and Kg. Manggut, Sarawak ($p>0.05$) (Table 1). The muscle of *X. naritus* collected from Kg. Manggut showed higher level of crude fat content than from Kabong, Sarawak (Table 1). However, the crude fat content of the muscle of *X. naritus* between Kg. Manggut (0.49%) and Kabong (0.47%) was not significantly different ($p>0.05$) (Table 1). There were significant difference ($p<0.05$) of crude fibre and ash content between the samples from Kabong and Kg. Manggut. The crude fibre and ash contents in the muscle of *X. naritus* from Kg. Manggut also were higher than Kabong (Table 1).

Toxin analysis

The results of TTX concentration (µg/g) in the muscle of *X. naritus* from Kg. Manggut and Kabong, Sarawak are shown in Table 2. The TTX concentration (µg/g) in the muscles of *X. naritus* from Kg. Manggut, Sarawak was significantly higher than Kabong's specimens ($p<0.05$). There was significance different in the mean TTX concentrations (µg/g) in the muscles that dissected between the skilled and unskilled person ($p<0.05$). The mean TTX concentrations (µg/g) in the muscles that dissected by the skilled person showed significantly lower than that of the unskilled person for both locations ($p<0.05$). The mean TTX concentration (µg/g) in the muscle which dissected by the skilled person was 10.17 µg/g and 6.63 µg/g from Kg. Manggut and Kabong respectively. While the muscles from Kg. Manggut and Kabong that dissected by the unskilled person showed significantly higher mean TTX concentration (65.33 µg/g and 29.07 µg/g respectively; $p<0.05$).

Discussion

In this study, the specimens of yellow puffer fish *X. naritus* that collected from Kabong and Kg. Manggut, Sarawak were not divided according to their sex. The yellow puffer fish that collected from Kabong, Sarawak were larger and heavier than the specimens from Kg. Manggut, Sarawak. In the previous study, the size of female specimens (13.0-31.0 cm) was larger than male specimens (12.5-22.5 cm) (Mohd Nor Azman *et al.*, 2013). Imelda *et al.* (2012) reported that the total length in the female yellow puffer fish ranged between 21.6 cm and 33.9 cm compared to the male's size ranged between 11.3 cm and 19.8 cm. While, Mohamad *et al*. (2008) observed the range total length in six samples of yellow puffer fish was between 19.6 cm and 26.9 cm. However, the maximum body weight of puffer

Table 1 Proximate composition of the muscle of *X. naritus* from Sarawak (% of dry weight)

Sampling location	Kg. Manggut		Kabong	
Number of specimens	18		18	
	Mean±SD	Min-Max	Mean±SD	Min-Max
Moisture (% ww)[a]	77.36±1.13[a]	75.23-78.93	79.97±0.56[b]	78.59-80.63
Crude protein (% dw)[b]	87.91±2.94[a]	84.4-92.8	88.22±2.58[a]	84.6-92.3
Crude fat (% dw)	0.49±0.26[a]	0.01-0.77	0.47±0.17[a]	0.24-0.77
Crude fibre (% dw)	0.44±0.16[a]	0.30-0.78	0.25±0.16[b]	0.09-0.58
Ash (% dw)	5.48±0.05[a]	5.4-5.6	5.08±0.19[b]	4.7-5.5

[a]ww – wet weight
[b]dw – dry weight
Data are shown as mean±standard deviation (SD)
Different alphabetical superscripts indicate difference between the measured values in each row ($p<0.05$)

Table 2. TTX concentration (µg/g) of the muscle of *X. naritus* from Sarawak

Sampling location		Kg. Manggut		Kabong	
Dissecting technique		skilled	unskilled	skilled	unskilled
Number of specimens		26	30	20	18
	Total	56		38	
Body length (cm)		16.9±3.25[a]	19.1±4.25[a]	23.8±2.69[b]	22.6±1.48[b]
	Range	13.4-28.0		17.8-28.8	
	Total	18.1±3.93[a]		23.2±2.26[b]	
Body weight (g)		111.9±96.2[a]	162.8±142.1[a]	282.3±90.8[b]	307.3±50.1[b]
	Range	42.6-492.5		138.1-489.4	
	Total	139.2±124.5[a]		294.1±74.5[b]	
TTX (µg/g)		10.17±1.90[b]	65.33±1.87[d]	6.63±1.48[a]	29.07±2.37[c]
	Total	24.1±3.09[a]		13.4±2.69[b]	

Data are shown as mean±standard deviation (SD)
Different alphabetical superscripts indicate difference among the measured values in each column ($p<0.05$)

fish observed in this study (492.5 g and 489.4 g; Kg. Manggut and Kabong respectively) was higher than that of 190.7 g and 206 g reported by Imelda *et al.* (2012) and Mohd Nor Azman *et al.* (2014) respectively but lower than reported by Mohamad *et al.* (2008) (533.8 g) and Mohd Nor Azman *et al.* (2013) (711 g). According to Aydin (2011), length and weight are regarded as important growth criteria in the ecology of fish.

In this study, the moisture content of the muscles from Kg. Manggut was between 75.23% and 78.93%, and from Kabong was between 78.59% and 80.63%, respectively. These values were in the range of values that usually found in other commercial fish from the West coast of Peninsular Malaysia (Nurnadia *et al*., 2011). The values were slightly higher to that of farmed puffer fish muscles of *Fugu obscurus* (76.9%), *Fugu flavidus* (78.0%) and *Fugu rubripes* (77.3%) (Tao *et al*., 2012) and wild *Lagocephalus sceleratus* (78.5%) (Aydin *et al*., 2013).

The crude protein contents (wet weight) of the muscles from Kg. Manggut (19.9% ww) were much higher than that muscle of farmed *F. obscurus* (18.4% ww), *F. flavidus* (18.1% ww) and *F. rubripes* (17.8% ww) but slightly lower than wild *L. sceleratus* (21.62% ww) (Tao *et al.*, 2012; Aydin *et al.*, 2013). While Saito and Kunisaki (1998) found lower protein (16.5% ww) for wild and cultured puffer fish *T. rubripes*. These variations might be due to the environmental condition and consumption pattern of the fish (Eswar *et al.*, 2014).

In general, the muscle of *X. naritus* collected from Kg. Manggut showed higher level of crude fat, crude fibre and ash content than from Kabong, Sarawak. The difference might be due to the specimens were obtained from different locations and habitats. The specimens from Kabong were caught in estuaries of Sungai Krian, while the specimens from Kg. Manggut were collected from Batang Saribas. Other puffer fish species showed higher fat contents for both wild and cultured samples. Saito and Kunisaki (1998) found 0.7% and 0.9% fat composition for wild and cultured *T. rupripes* respectively. While Koizumi and Hiratsuka (2009) reported fat content for cultured and wild *T. rubripes*, as 0.84-0.96% and 0.87-1.01% respectively. The differences and variations in proximate compositions might be due to geographical location, species type and seasonal effects (Aydin *et al.*, 2013).

The results from this study indicated that the muscles of *X. naritus* from both locations were toxic and the distribution of the toxin was unequal. The muscles of *X. naritus* from Kg. Manggut, Sarawak showed higher TTX concentration (µg/g) compared to the same species collected from Kabong, Sarawak. The same species contained different toxin levels could be attributed to the fact that the fish were caught from different areas and habitats (Mohd Nor Azman

et al., 2014). Puffer fish from different habitats might consume different levels of diet in their habitats. The feed consumed might also contribute to the level of TTX in the tissues of puffer fish. According to Noguchi *et al.* (2006b), marine puffer fish are believed to accumulate TTX through the food chain by ingesting TTX-bearing organisms such as starfish, gastropods, crustacean, flatworms, ribbon worms and so forth. The TTX concentration of the muscle recorded in this study was higher than the previous study (Mohd Nor Azman *et al*., 2013; 2014). In our previous study, the level of TTX was varied among the individual puffers and time of collection (Mohd Nor Azman *et al.*, 2013). However, Che Nin *et al*. (2010) reported that TTX was not detected in the muscle of yellow puffer fish from Sampadi, Kuching, Sarawak. Generally, the toxicity of puffer fish has been reported to show large individual, regional and seasonal variations (Kungsuwan, 1994; Nagashima *et al.*, 2001; Yu and Yu, 2002; El-Sayed *et al.,* 2003; Noguchi *et al*., 2006a; Rodriguez *et al.*, 2012).

Based on this study, the amount of TTX in the muscles that dissected by the unskilled person was higher than the skilled person. The results obtained indicated that the muscle, the edible part of fish that dissected by the skilled person contained 6.63-10.17 µg/g of TTX and 29.07-65.33 µg/g of TTX by the unskilled person which can be classified as weakly and moderately toxic, respectively (Noguchi *et al.,* 2006; Sabrah *et al*., 2006). The minimum lethal dose and minimum acute dose of TTX to humans (wt. 50 kg) were estimated to be around 2 mg (10,000 MU) and 0.2 mg (1000 MU), respectively (Katikou *et al*., 2009). According to Mahmud *et al.* (2001), muscle in many toxic species was regarded as edible. Although *X. naritus* is harmless to humans and safe to eat but the present study has shown relatively high levels of TTX in the muscle (Froese and Pauly 2011).

Food poisoning has been reported from different geographical regions due to ingestion of puffer fish and lethality depended on the amount of TTX present in the consumed fish tissues (Chou *et al*., 1994). The results from this study showed that even though the muscle that prepared by the skilled person was classified as weakly toxic, it could be considered unsafe for human consumption if more than 1 g is consumed which was above the regulatory limit (2 µg/g) (Japan Food Hygiene Association 2005) However, it can vary depend on age, health and sensitivity to the toxin (Cohen *et al*., 2009). Nevertheless, it is safe for consumption if the poisonous parts were removed thoroughly. The fish had to be prepared properly and completely cooked. According to Gambang and Lim (2004), some of the local people living in the middle regions of Batang Saribas, Betong Division, Sarawak had experiences and skills with the preparation of yellow puffer fish as they have eaten the fish for generations. According to Parvaneh *et al.* (2012), there are three methods to prepare the yellow puffer fish by the local people such as chopping, scissor cutting and traditional methods, but the concept remained the same. In Japan, only chefs who have been trained and licensed can prepare or serve puffer fish to the public and it is strictly controlled by law (Cohen *et al*., 2009).

Conclusion

This study showed that muscle of *X. naritus* collected from Kg. Manggut and Kabong, Sarawak contained high amounts of moisture and crude protein contents. While the crude fat, crude fibre and ash contents were low. The amount of TTX in the muscle of *X. naritus* from Kg. Manggut was higher than Kabong. The amount of TTX was lower in the muscle that prepared by the skilled person from both locations. However, the amount of TTX in the muscle was found to be toxic for human consumption as the TTX level was above the regulatory limit (2 µg/g) (Japan Food Hygiene Association 2005). To our knowledge, this is the first report to determine the proximate composition from the muscle of yellow puffer fish from Sarawak. The proximate values obtained from this study shows that the yellow puffer fish *X. naritus* are good protein resources and can be considered for human consumption. The information given in this study could be used as a guide on the amount of yellow puffer fish that can be consumed safely if prepared in a proper way. It is essential to advice and educates the local population of Sarawak of the potential health risk of puffer fish consumption if not carefully prepared as the TTX is heat-resistant and not destroyed by freezing or cooking (Noguchi and Ebesu, 2001). This study indicates the importance removing TTX to ensure the safe consumption of puffer fish and to prevent from the occurrence of puffer fish poisoning.

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