Characteristics of chemical compounds of horseshoe crabs
*Tachypleus gigas* in different body proportions

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Abstract. Horseshoe crabs are a marine animal that has been widely used empirically, but in Indonesia the exact bioactive compounds are unknown. People poisoning caused by consuming horseshoe crabs has also been reported. The aims of this study was to observe the bioactive compounds in every part of horseshoe crabs which suspected to be related to toxicity. The work was conducted to measure morphometrics, to count body proportions, and to determine phytochemical compositions, toxicity, and antioxidants in different body proportions. Female horseshoe crabs with average weight more than 500 g were obtained from the waters of Lamongan Regency in July 2018. The highest proportion of the different body parts was the shell and the lowest was viscera. Edible portions of horseshoe crabs were 45.39%. Meat contains low fat 1.97% and high fat in gonads 4.87%. The solvent that produces the best extracts from the gonads was ethanol and used to extract meat, viscera, and gills. Viscera extracts were toxic with values of LC50 109.44 ppm, gonad and meat extracts have not shown to be toxic with values of LC50 1251.30 and 1101.79 ppm. Antioxidant activity of meat is weak (164.50±23.16 ppm), and gonads are very weak (330.47±02.60 ppm).

Keywords: active compound, characterization, extract, horseshoe crabs, morphometric, yield

1. Introduction

The Indo-Pacific Waters which cover Indonesian waters are rich in aquatic resources (Lasabuda 2013). One of the faunal resources are horseshoe crabs, consist of three out of four global species in Indonesian waters, e.g. *Carcinoscorpius rustondicauda*, *Tachypleus gigas*, and *T. tridentatus*. In 2014 those species are in treated status and in 2019 stated as data deficient (IUCN 2019, Meiliana et al 2016).

Takahashi (2016) and Smith et al (2016) cited several research works that in the USA, the utilization of horseshoe crabs started for fertilizer across their range along the Atlantic Coast of the U.S. from Maine to Florida (Shuster 2003), as bait for eel, whelk and conch fisheries in several states, and their blood is collected (with individuals returned to the wild after bleeding) for the biomedical industry. The harvest of horseshoe crabs was largely unregulated until 1998 when the horseshoe crabs Fishery Management...
Plan was developed by the Atlantic States Marine Fisheries Commission, which enforced state by state
harvest quotas beginning in 2001 (ASMFC 2013). In response to declining horseshoe crabs populations,
states throughout the Atlantic Coast enacted restrictions on harvest such as the moratorium on harvesting
female crabs in Delaware. In 1991, South Carolina became the only state to completely prohibit
horseshoe crabs harvest for the bait industry (ASMFC 2013). However, horseshoe crabs are still
collected for the biomedical industry in South Carolina.

In Indonesia, the utilization of horseshoe crabs meat and gonads are limited in several coastal
communities due to the toxic substances contained in flesh and gut, which well known by the fishers
(Pratiwi 1993). Beside as food, the horseshoe crabs meat is used as bait of saltwater eel (*Euristhmus
microcephs*) (Rubiyanto 2012). However, people poisoning always happens (reported or unreported) after
eating the horseshoe crabs. Several research works have been conducted in Indonesia connected to the
horseshoe crabs, i.e. blood plasma utilization as inhibitor of negative and positive bacteria growth, meat
characterization of different habitat of *Carsinoscorpius rotondicauda* which contain different degree of
terpenoid, steroid, and zoosterol (Asih *et al* 2018).

As frequently and incidentally caught by the fishers, the local horseshoe crabs characterization was
needed to be observed to get in-depth information concerning its toxicity. The result was expected useful
for the local fishers and coastal communities which part of horseshoe crabs body was edible to be
consumed and how much its the daily intake.

2. Materials and methods

2.1. Materials

The samples were taken from Tangsi Sidokumpul market at Lamongan. The sample were selected only
the female *Tachypleus gigas* weight from 500˗900 g each (the female horseshoe crabs is consumed by
local communities especially the eggs). The live sample was packed on styrofoam with one-third volume
of seawater then carried to laboratory in the Faculty of Fisheries and Marine Science Dramaga Campus
in Bogor after land traveling 12 hours. In the laboratory, the horseshoe crabs were prepared to divide
each part of the body into meat, gonad, viscera, and gills. The toxicity test used larvae of *Artemia salina*.

Chemical material used were: ethanol 96%, ethyl acetate 100%, and n-hexana 99.5%, chloroform,
ammonia, H$_2$SO$_4$ 2M, methanol 70%, NaOH 10%, FeCl$_3$ 5%, H$_2$SO$_4$ concentrated, DPPH (Sigma).
Reagents: Meyer, Wagner, Dragendroff. Research devices were used: analytical scale (Sartorius TE 64,
Germany), a multiwell plate, orbital shaker (*WiseShake SHO-2D*, Korea), spectrophotometer UV-Vis
1259 (*Labomed 23RS*, California, USA).

2.2. Methods

2.2.1. Morphometric measuring (Yamasaki *et al* 1998). The samples were morphometrically
measured as shown in figure 1.

2.2.2. The yield percentage. The yield of each part of horseshoe crabs was calculated by dividing the
weight of each body part with total weight of samples.

2.2.3. Proximate analysis. Proximate analyses were done using methods of AOAC (2005), measuring
protein, lipid, moisture, and ash content.

2.2.4. Solvent screening (*Binsan* *et al* 2008). The horseshoe crabs gonad were macerated in ethanol
96%, ethyl acetate, and n-hexane 99.5%. The maceration used 1:5 (sample:solvent) ratio (w/v) on 180
rpm for 1 hour. The chosen solvent was then used as a solvent for the meat, gonads, vicera, and gills
maceration.
2.2.5. Extraction of bioactive compounds (Binsan et al 2008). As many as 25 g of samples (gonads, meat, viscera, gills) were chopped, soaked in 125 mL ethanol (1:5 w/v) and macerated at room temperature using orbital shaker 180 rpm for 1 hour. The maceration yield filtered by using Whatman paper no.42. The filtrate was evaporated using rotary evaporator at 40°C.

2.2.6. Toxicity test BSLT (Krishnaraju et al 2005). In vitro toxicity test using brine shrimp lethality test (BSLT) method to the larvae of *Artemia salina*.

2.2.7. Phytochemical Analysis (Harborne 1987), consist of alkaloid, steroid, triterpenoid, saponin, phenol hydroquinone, and flavonoid.

2.2.8. DPPH antioxidant test (Boeing et al 2014 with modification). The gonad extract was diluted in series of ethanol with a concentration of 50, 75, 100, 125, and 150 ppm, while for meat extract used 100, 200, 300, 400, and 500 ppm of ethanol. Ascorbic acid and positive control were used as comparative treatment, those diluted in ethanol p.a with concentration 1, 2, 3, 4, 5 and 6 ppm. As much as 4.5 mL of each solution (extract, control, and ascorbic acid) were taken and added 500 μL DPPH solution 0.1 mM at different reaction tubes. The reaction was left for about 30 minutes at a dark room with 37°C temperature. The absorbance of samples was read using UV-Vis spectrophotometer at 517 nm wavelength. The blank solution was made from 4.5 mL of ethanol and added 500 μL DPPH solution.

2.3. Data analysis

The work has been carried out using a completely randomized design with double replication. Data of morphometric, proximate, and phytochemical were presented the mean and its standard deviation with descriptive discussion. Others data were statistically analyzed with ANOVA using tool Statistical Product and Service Solutions (SPSS) 21. Antioxidant activity data of gonads and meat were analyzed using T-test.

3. Results and discussion

3.1. Morphometric and body proportion of horseshoe crabs

The data was shown in table 1. Sumarmin et al (2017) work indicated that female horseshoe crabs are bigger than males. The body proportion of horseshoe crabs consisted of 44.03% carapace, 34.34% gonads, 10.91% meat, 8.40% gills, and 2.33% vicera.
Table 1. Morphometric data of female horseshoe crabs.

| Parameter             | Average       |
|-----------------------|---------------|
| Total weight (g)      | 643.00±96.89  |
| Total length (cm)     | 39.01±04.31   |
| Standard length (cm)  | 19.47±02.70   |
| Prosoma width (cm)    | 20.47±02.14   |
| Prosoma length (cm)   | 11.47±01.61   |
| Opisthosoma length (cm)| 8.00±01.11   |
| Telson length (cm)    | 19.54±03.15   |

3.2. Chemical composition of gonads and meat

The edible portion of horseshoe crabs *Tachypleus gigas* consist of gonads and meat, reach 45.39±0.02%. Chemical composition of horseshoe crabs meat and gonads are shown in table 2. Ash content of horseshoe crabs *T. gigas* meat and gonads were higher than what was presented in swimming crabs and crabs, in contrary with higher lipid contents than swimming crabs and crabs. Fish was classified into three groups depend on their lipid content: low (2-3%), medium (3-5%), and high (6-10%), so horseshoe crabs were grouped into low lipid products.

Table 2. Chemical composition of horseshoe crabs meat and gonads.

| Parameter   | Horseshoe crabs | Crabs<sup>a</sup> | Horseshoe crabs | Crabs<sup>a</sup> | Swimming crabs<sup>b</sup> |
|-------------|-----------------|-------------------|-----------------|-------------------|-----------------------------|
| Water       | 61.66±0.34      | -                 | 79.24±0.12      | 82.68             | 81.27                       |
| Ash         | 0.68±0.04       | -                 | 0.55±0.20       | 2.45              | 1.82                        |
| Lipid       | 4.87±0.06<sup>c</sup> | (12.72±0.37)<sup>c</sup> | 0.16<sup>c</sup> | 1.97±0.10         | 0.28                        |
| Protein     | 26.10±0.31      | (68.07±0.25)<sup>c</sup> | 88.55<sup>c</sup> | 14.44±0.12        | 11.90                       |
| Carbohydrate| 6.51±0.14       | -                 | 3.75±0.10       | 2.69              | 0.39                        |

Source: <sup>a</sup>Sulaiman and Hanafi (1992); <sup>b</sup>BBPMHP (1995); dry basis n =2

3.3. Rendemen of *T. gigas* gonads

The yield of gonads extracted by different solutions is shown in table 3. The anova analyses (<i>p</i> < 0.5) indicated that different solutions influenced to the extracted rendemen of the gonads. The Duncan test has shown that the ethanol maceration process resulted in significantly more rendemen compared to two others solution (ethyl acetate and n-hexane). This indicated that the polarity of ethanol more suitable (like dissolves like) for the extraction of active compounds on the samples compared to ethyl acetate and n-hexane. Ethanol is a solution which has a higher polarity index compared to ethyl acetate and n-hexane; this means the ability of ethanol to penetrate the gonads and meat tissues are better than ethyl acetate and n-hexane. Other advantages of using ethanol for the maceration process is suitable to extract polyphenol compound and (relatively) safe to drugs (Tiwari <i>et al</i> 2011).

Table 3. The yield of extracted gonad using different solutions.

| Solution      | Extract Yield (%) | Form   | Colour     |
|---------------|-------------------|--------|------------|
| Ethanol       | 4.16±0.13<sup>b</sup> | Paste  | Brown      |
| Ethyl Acetate | 0.94±0.06<sup>a</sup> | Paste  | Brown      |
| n-Hexane      | 0.84±0.10<sup>a</sup> | Paste  | Yellowish-brown |
3.4. Extracted rendemen of T. gigas bodies
Ethanol was used to extract the meat, gills, and viscera. The results are shown in table 4. The polar solution could extract alkaloid compound, phenolic component, tannin, sugar, amino acid, and glycoside (Harborne 1987). The extract’s color seem different in between samples, it might be influenced by different bioactive contents, chemical and physical characteristics of sample tissues.

| Sample   | Extract Yield (%) | Form   | Colour        |
|----------|-------------------|--------|---------------|
| Gonads   | 4.16±0.13<br><sup>b</sup> | Paste  | Brown         |
| Meat     | 1.70±0.04<br><sup>a</sup> | Paste  | Yellowish-brown |
| Gills    | 1.20±0.07<br><sup>a</sup> | Paste  | Brown         |
| Viscera  | 1.50±0.08<br><sup>a</sup> | Paste  | Blackish brown |

3.5. Toxicities of T. gigas extract
In vitro toxicity test was used to predict the contents of toxins in a material (Pratama et al 2014), one of the methods was BSLT (brine shrimp lethality test) which was an initial step on a toxicity test LC<sub>50</sub> (lethal concentration), is a dose of active compound which can kill 50% of shrimp larvae during 24 hours incubation (Lisdawati et al 2006). A material could be classified as very toxic if the LC<sub>50</sub> value less than 30 ppm, LC<sub>50</sub> 31-200 ppm is toxic, LC<sub>50</sub> 201-1000 ppm is low toxicity, and LC<sub>50</sub> more than is 1,000 ppm nontoxic (McLaughlin et al 1998). The result of BSLT test of ethanol extracted was shown in table 5.

| Extract Type | LC<sub>50</sub> (ppm)       | Category        |
|--------------|---------------------------|-----------------|
| Gonad        | 1,101.79±41.27<br><sup>c</sup> | Nontoxic        |
| Meat         | 1,251.30±125.16<br><sup>c</sup> | Nontoxic        |
| Gills        | 533.21±55.15<br><sup>b</sup> | Low toxicity    |
| Viscera      | 190.44±13.79<br><sup>b</sup> | Toxic           |

Types of toxins contained on T. gigas samples were tetrodotoxin (TTX) (Kungsuwan et al 1987). Work of Tanu and Noguchi (1999) stated that T. gigas (average weight 227.9 g) in Bangladesh and Thailand were nontoxic, while other species of horseshoe crabs, like Carcinoscorpius rotundicauda females from Bangladesh during November-December 1998 have shown low toxicity (viscera 2,200-3,900 ppm, gonads 5,200-7,400 ppm). TTX was produced by Vibrio spp. which live in its intestine and was transferred to the gonads during the maturation process (Kungsuwan et al 1987). A vital dose of TTX for human was approximately 10,000 ppm per kg of body weight, consuming 100 g of horseshoe crabs meat. This could have highly negative impact on consumers health (Ngy et al 2007). TTX could be bioaccumulated through body food chain system (Williams 2010).

3.6. The active component of T. gigas ethanol extract
Gonads crude extract contains alkaloid, saponin, flavonoid, and steroid, while from meat extract get saponin, tannin, steroid, and phenol. From gill extracts alkaloid, saponin, flavonoid, steroid, and phenol, were found while from viscera extract there are alkaloid, saponin, flavonoid, triterpenoid, a phenolic were isolated. Marine fauna which commonly contains steroids is a group of crustacea when there are in the molting process. The component contained in the samples is shown in table 6.

3.7. Antioxidant activity of as T. gigas’ gonads and meat
The DPPH’s test indicated that the IC\textsubscript{50} of ascorbic acid was 1.42±0.02 ppm, while the gonad 330.47±2.6 ppm and meat 164.5±23.16 ppm. The smaller the value of IC\textsubscript{50} detected from a sample the stronger the antioxidant activity will be. The antioxidant activities of extracted gonads and meat are influenced by the contained active compounds. Phenol and flavonoid could retard the free radical compound (Hafiluddin et al 2011). Antioxidant activities which contained in the crude extracts of gonads and meat were also influenced by saponin an antioxidative compound (Xiong and Sheen 2012).

Table 6. Phytochemical test results of extracted ethanol horseshoe crabs meat.

| Components  | Extract |
|-------------|---------|
|             | Gonads | Meat | Gills | Viscera |
| Alkaloid a  | +      | -    | +     | +       |
| Alkaloid b  | +      | -    | +     | +       |
| Alkaloid c  | +      | +    | +     | +       |
| Saponin     | +      | +    | +     | +       |
| Flavonoid   | +      | -    | +     | +       |
| Steroid     | +      | +    | +     | +       |
| Triterpenoid| -      | -    | -     | +       |
| Phenolic    | -      | +    | +     | +       |

+ = detected; - = undetected

4. Conclusion
From the size of T. gigas 643 g weight and 39.01 cm length, the most abundant part was carapace. The edible (gonad and meat) yield was 45.39%. Ethanol was the best solution for extracting active compounds from the samples. The viscera were categorized as a toxic substant, gonads and meat were categorized as nontoxic. The lipid of meat was classified as low, while in the gonad the lipids were medium. The antioxidant activity of the meat was weak and in the gonads it was even weaker.

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