

Biomaterial compounds and bioactivity of horseshoe crab *Carsinoscorpius rotundicauda* biomass harvested from the Madura Strait

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Abstract. *Carsinoscorpius rotundicauda* or horseshoe crab biomass has great potential in pharmaceutical aspects, one of them as an antibacterial substance. Information related to the benefits of *Carsinoscorpius rotundicauda* biomass such as meat and blood is essential because in fact, this species is considered a pest by fishermen, a low market value and has no legal protection in Indonesia. The purpose of this study was to determine the content of biomaterial compounds of meat and bioactivity of *Carsinoscorpius rotundicauda* plasma on bacterial inhibition from three different stations harvested from the waters in Madura Strait. The observation of the utilization of the potential from horseshoe crab biomass ie meat and plasma was performed by measuring the content of biomaterial compound in horseshoe crab meat by HPLC method and zone of inhibition test for gram-positive and gram-negative bacteria in horseshoe crab plasma. Analysis of the relationship between the two parameters used the Principal Component Analysis. The highest content of biomaterial compounds of monoterpenoid and zoosterol is found in horseshoe crab from Bangkalan waters, namely monoterpenoid (18.33 ppm) and zoosterol (22.67 ppm), while the smallest compound content obtained in horseshoe crab from Probolinggo waters, namely monoterpenoid (13.67) ppm and zoosterol (17.33 ppm). The bioactivity of Dark Blue Plasma (BDP) and Light Blue Plasma (LBP) samples of horseshoe crab obtained around the Madura Strait has the ability to inhibit gram-positive bacteria higher than gram-negative bacteria. The total average of DBP plasma inhibitory power on *Staphylococcus aureus* was 10.00 mm and 10.07 mm on *Bacillus*, while that in LBP sample, *Staphylococcus aureus* was 9.11 mm and *Bacillus* was 9.67 mm. The high biomaterial compound content of horseshoe crab is in line with the ability of horseshoe crab plasma to inhibit *Bacillus* and *Staphylococcus aureus*.

1. Introduction

Horseshoe crab is a unique animal as it morphologically resembles a spider, has a shell, and live in the sea. There are 4 species remaining that identified belongs to the classification system of horseshoe crab in the world's, namely *Limulus polyphemus* (Linnaeus, 1758), *Tachypleus gigas* (Muller, 1785), *Tachypleus tridentatus* (Leach, 1819), and *Carcinoscorpius rotundicauda* (Latreille, 1802). *Carcinoscorpius rotundicauda* or mangrove horseshoe crab belongs to the class *Merostomata*, an



ancient class of animals, and ordo *Xiphosurida* which have smaller body size than other types of horseshoe crab [1]. Horseshoe crab is spread in some intertidal waters of Asia and America. These fossil organisms are distributed in Asian waters including Hong Kong to China waters [2], Singapore waters [3], and Malaysian waters [4]. This species is also found around the Madura Strait (Indonesia), namely Bangkalan waters [5], Sumenep waters [6] and Gresik waters up to Surabaya waters [7].

Horseshoe crab plays important roles in the intertidal ecosystem and community life such as the fulfillment of food and medicine. Eggs and meat horseshoe crab are used as expensive proteins in Malaysia and Thailand [8]. The nature of horseshoe crab which selective in choosing food has the potential to maintain the configuration of organism in the mangrove ecosystem in order to avoid blooming [9]. Horseshoe crab is also widely used as a traditional and modern pharmaceutical ingredient. Utilization of biomass and biomaterials from horseshoe crab in the form of meat (flesh) and blood as a modern pharmaceutical ingredient has been developed in America since the 20th century. The need for LAL extract (Limulus Amebocyte Lysate) from horseshoe crab plasma biomass *Limulus polyphemus* (endemic species of America) as a bacterial endotoxin test for medical devices and drug testing of about 500.000 liters annually gives a profit of about 50 million USD/year [10]. The same potential is also seen in *Carcinoscorpius rotundicauda*. Plasma from *Carcinoscorpius rotundicauda* biomass is used by Indian society to cure joint pain [11]. Plasma biomass extracts of these organisms are able to neutralize tetrodotoxin in neuroblastoma cells tested in mice [12]. Several studies have shown that plasma horseshoe crab type *Carcinoscorpius rotundicauda* contains haemagglutinin compounds [13], tachyplesia [14], C-reactive protein 2 [15], which acts as an active ingredient for inhibiting antibacterial activity and agglutination. The carapace extract of this species is also potentially used as antifouling and antibacterial [16].

The protection status of horseshoe crab in Indonesia is listed in Peraturan Pemerintah No. 7 tahun 1999 which mentions that *Tachypleus gigas* belongs to the category of endangered marine animals [17]. *Carcinoscorpius rotundicauda* is a species that is not yet protected. The absence of laws to protect this species may lead to the potential for population decline due to unintentional exploitation by the community because it is considered pests and has no economic value, so it is generally killed by most fishermen when it caught by nets. The habit of killing horseshoe crab mangrove when caught by the net makes this species untapped and wasted. This phenomenon can indirectly disrupt the population of horseshoe crab in the location, so it is necessary to study the utilization of this organism biomass so that people know the importance of horseshoe crab one of them in the pharmaceutical field such as the potential of biomaterial compounds and bioactivity. The purpose of this study was to analyse the content of bioactive compounds of horseshoe crab from three different locations harvested from the Madura Strait and to know the plasma bioactivity of horseshoe crab against gram-negative and gram-positive bacteria.

2. Materials and Methods

2.1. Materials and Tools

Materials used in this research is nets, calipers, label, autoclaves, petri disk, tweezers, micro pipettes, bunsen, incubators, paper discs, gloves, 1 set of syringes, falcon tubes, oven, pipette drops, measuring tube, erlenmeyer, centrifuge, HPLC (High Performance Liquid Chromatography), refrigerators, knives, aquades, aluminum foil, plastic bag, tissue, methanol, biomass of horseshoe crab ie meat and plasma (blood or hemolymph) *Carcinoscorpius rotundicauda*, spirits, alcohol, ampicillin, tetracycline, *Escherichia coli*, *Staphylococcus aureus*, *Vibrio harvey*, *Bacillus*, TSA (Tryptone Soya Agar), NB (Nutrient Broth).

2.2. Determination of station and Sampling

This research was conducted from October 2016 until January 2017. Sampling was conducted at 3 stations in Madura Strait namely Kertasada-Sumenep waters, Dakiring Socah-Bangkalan waters, and RanduPutih-Probolinggo waters (Figure 1). Samples were obtained by spreading 3 belt transects in the form of nets with the size of 200 meter x 1 meter with a mesh size of 3 inches at 3 stations. The transect

is extended parallel to the coastline and the distance of each sub-station is 150 meter. The sample caught was taken to the laboratory with alive conditions for further analysis.

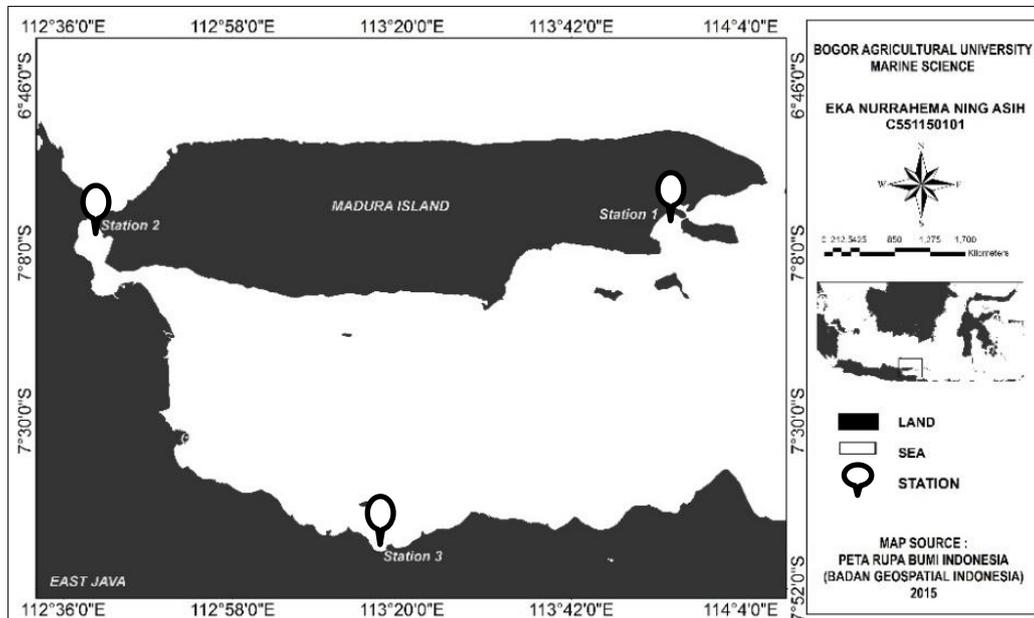


Figure 1. Research Sites in Madura Strait Waters.

2.3. Estimation of Biomaterial Compounds

2.3.1. Criteria of test samples

Morphometric measurements in horseshoe crab were performed by measuring the width of the horseshoe crab's prosoma using the sliding term (calipers). Horseshoe crab sample criteria used for testing of biomaterial compounds was female horseshoe crab with a maximum width of prosoma of 12 cm (Figure 2), which is numerous width captured by the net in three research sites. On this size, horseshoe crab considered as the mature at the waters of the Madura Strait.

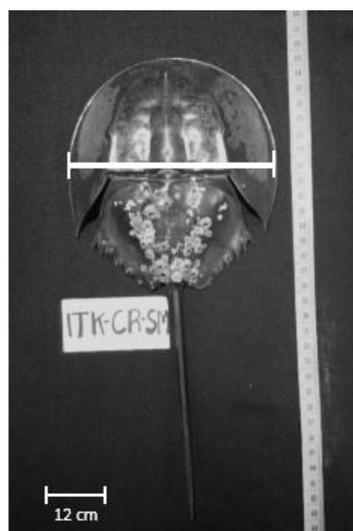


Figure 2. The size of *Carinoscorpius rotundicauda* prosoma

2.3.2. Biomaterial compounds test

The biomaterial compound test was performed by measuring the levels of secondary metabolite compounds or bioactive compounds including the terpenoids and steroids according to the Harborne method (1996) [18]. The sample preparation was done by weighing 10 grams of horseshoe crab meat biomass *Carsinoscorpius rotundicauda*, then put into erlenmeyer with 100 ml of methanol. Horseshoe crab meat biomass that has been mixed with methanol is smoothed using a mini blender. The fine sample was then eluted using a methanol solution in the preparative column to bind the fats and proteins contained in the solution. The eluted sample was then refluxed using methanol at 50°C for 72 hours. The refluxed sample was filtered using a 0.45 micron filter to separate residues and filtrate. The filtrate obtained was centrifuged at a rate of 5000 rpm for 5 min to separate the supernatant and natan contained in the filtrate. The supernatant was kept at 4°C for further analysis. The readings of terpenoids and steroids are performed using HPLC. The reading of terpenoid content is conducted by taking a sample solution of 0.5 ml plus 10 ml of methanol using an injector made of stainless copper. The solution is injected directly into HPLC in the mobile phase with λ maximum of 230 nm dynamic column C18 UV VIS detector flowing under pressure to the column and mobile phase λ maximum 254 nm dynamic column C18 UV VIS detector .

2.4. Bioactivity Test

2.4.1. Plasma of horseshoe crab

The sampling biomass of *Carsinoscorpius rotundicauda* blood plasma as the sample of the antibacterial test was performed according to Derek method (1993). The samples of horseshoe crab from each research site were sterilized with sterilized water and alcohol. Biomass of plasma was collected by injecting the cardiac tissue around the prosoma and opisthosoma muscles. The biomass plasma collection was taken from several horseshoe crab samples which then stored in a sterile polypropylene tube. The plasma was then centrifuged at 4×6000 rpm for 1 hour at 4°C to separate the supernatant (liquid plasma of horseshoe crab) and pellet (the settling cell). Plasma was stored at -20°C, then tested on agar media to observe the inhibition zones against gram-negative and gram-positive bacteria.

2.4.2. Inhibition zones of the bacteria

Gram-negative bacteria used for the inhibition zone test were *Eschericia coli* and *Vibrio harveyi*, whereas the gram-positive bacteria used were *Staphylococcus aureus*, and *Bacillus*. The antibacterial activity test was done by using 3 stages that are media preparation to make Tryptic Soy Agar media, bacterial rejuvenation and bacterial inhibition test using Nutrient Broth buffer media. The bacterial inhibition test was performed by inoculating the rejuvenated bacteria on the Nutrient Broth medium (the buffer medium used to reactivate the bacteria) and then homogenized them using vortex and incubated for 18 hours. Nutrient Broth which has contained bacteria on medium then placed for 1 hour at room temperature (27-28°C). The next stage was to put the paper disk then 20 micron of *Carsinoscorpius rotundicauda* plasma from each station was dropped in the center of the media. The last stage was done by reincubating the media for 24 hours then measured the inhibition zone. The bacterial inhibitory test was performed 3 replicates each (tryplo) on each test bacteria to see the resulting inhibitory zones

2.5. Data Analysis

Analysis of bioactive compounds and bioactivity of horseshoe crab from Kertasada-Sumenep waters, Socah-Bangkalan and Randuputih-Probolinggo waters were conducted using descriptive analysis. Data obtained during the study are presented in the form of tables and histogram graphs. The correlation of bioactive compounds and bioactivity of *Carsinoscorpius rotundicauda* from the three location sites was analyzed using Pricipal Component Analysis (PCA) with XLSTAT software 2016.2.01 [19].

3. Results and Discussions

3.1. Biomaterial Compounds from *Carsinoscorpius rotundicauda* Biomass

The identification of biomaterial compounds from the secondary metabolites of *Carsinoscorpius rotundicauda* meat biomass is the levels of terpenoids and steroids. Terpenoids are the largest secondary metabolite based on the amount of compounds and variations of their basic structural framework. These compounds are also produced by insects and marine organisms [20]. Terpenoids are produced by organisms as the synthesis result of acetic acid through a biochemical process with an enzymatic system or called metabolism [21]. Other biomaterial compounds also found in marine organisms are steroids. Steroids in marine animals are commonly found in many-legged animals (insect-shaped) having molting hormones [22] such as horseshoe crab. This biomaterial compound can be used as a drug substance [23].

The results of terpenoid and steroids tests obtained monoterpenoid and zoosterol compounds. The content of monoterpenoid and zoosterol compounds in horseshoe crab varies based on the study site (Figure 3). The highest content of *Carsinoscorpius rotundicauda* monoterpenoid and zoosterol is found in horseshoe crab existing in Bangkalan waters. Horseshoe crab in Bangkalan waters has monoterpenoid levels of 18.33 ppm and zoosterol of 22.67 ppm. The second highest biomaterial compound found in horseshoe crab living in Sumenep waters with monoterpenoid levels of 14.67 ppm and zoosterol levels of 20.33 ppm, while the smallest compounds of the three study sites found in horseshoe crab of Probolinggo waters with monoterpenoid and zoosterol levels of 13.67 ppm and 17.33 ppm respectively.

Differences in monoterpenoid and zoosterol levels of female horseshoe crab at three sites are might be caused by environmental conditions of horseshoe crab. Increased production of secondary metabolites by marine organisms is an attempt to protect themselves against environmental conditions, interactions with biotic and abiotic environments and chemical weapons against other organism disorders [24]. The high levels of monoterpenoid and zoosterol in *Carsinoscorpius rotundicauda* biomass from Socah-Bangkalan waters are suspected because this location has a higher environmental stress than the other two locations. Socah-Bangkalan waters nearby to the community settlement, Kamal waters and in direct contact with Gresik waters which is an area with dense industrial activity and ship repair area potentially effect the high concentration of aquatic pollutant such as harmful bacteria and heavy metal content.

Some research states that Socah-Bangkalan and surrounding waters condition as Gresik waters have high content of heavy metals in sediments and heavy metal content in water exceeds the standard quality threshold for continuity of marine organism by KMNKLH NO. 51 year 2004. The average weight of heavy metals on sediments in high Socah-Bangkalan waters ie Cd 0.36-0.38 mg/L, Cu 0.2 mg/L, Pb 47.31-50.49 mg/L, and Hg 9.01-9.07 mg/L [25]. The content of metal Pb in water at Manyar Gresik waters ranges from 0.31-0.57 mg/L and the metal content of Pb in the base of sediment ranges from 2.78-3.37 mg/kg [26]. The range can indirectly disrupt the physiological system of marine organism that performs an activity in sediments around Socah waters, one of which is *Carsinoscorpius rotundicauda*. Another indicator suggesting the high environmental stresses of horseshoe crab around this station is the abundance of coliform bacteria in the waters. The abundance of bacteria living on the profile of sea level around Kamal waters is $26-73 \times 10^6$ cfu/ml [27].

The average content of monoterpenoid and low horseshoe crab zoosterol was found in the Probolinggo sample. The low content of bioactive horseshoe crab from the sound of Randu Putih-Probolinggo because the condition at this location there is no pressure of heavy pollution and still feasible to survive horseshoe crab. The location of the Randu Putih-Probolinggo adjacent to Bentar beach in Gending sub-district is the gathering area of whale sharks [28]. The concentrations of metal Pb and Cd in Gending range between $<0.001-0.002$ mg/l, while the concentrations of Pb and Cd metal in the sediments are 0.09-0.53 mg/kg [29]. The concentration of the heavy metal is still below the limit by KMNKLH stipulated No. 51 of 2004 [30].

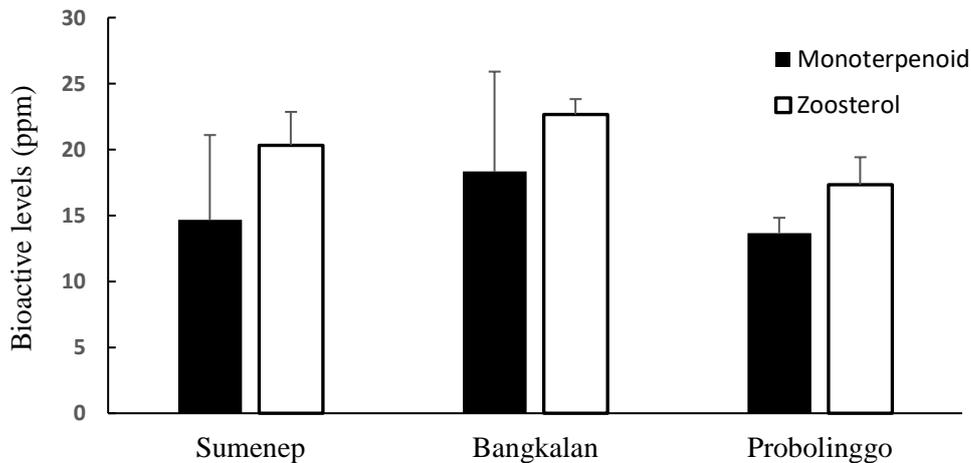


Figure 3. The average and standard deviation of biomaterial compound content of horseshoe crab based on study location.

3.2. Bioactivity Biomass from Plasma of *Carsinoscorpius rotundicauda* Against Bacteria

3.2.1. Extracted plasma of horseshoe crab

Liquid plasma containing hemocytes and protein [31]. Hemolymph or blood of horseshoe crab contains amebocyte, protein, sugar, salt, enzyme, creatinine, cholesterol and triglyceride cells [32]. Extracted plasma of *Carsinoscorpius rotundicauda* that centrifugation at 24.000 rpm is divided into 2 fractions namely light blue plasma and dark blue plasma. The light blue plasma is the top layer of plasma after dark blue plasma. Total plasma samples obtained from each study site were 35 ml, while the volume of light blue plasma sample is 25 ml (less than 10 ml compared to dark blue plasma) (Figure 4). The percentage value of both samples was 71% for dark blue plasma and 29% for light blue plasma samples. The protein content of two centrifugated plasma of horseshoe crab suggested vary, of which clear plasma of *Carsinoscorpius rotundicauda* (top layer) contains 0.6% protein of total crude plasma protein, while that bright blue plasma is 20% [33].

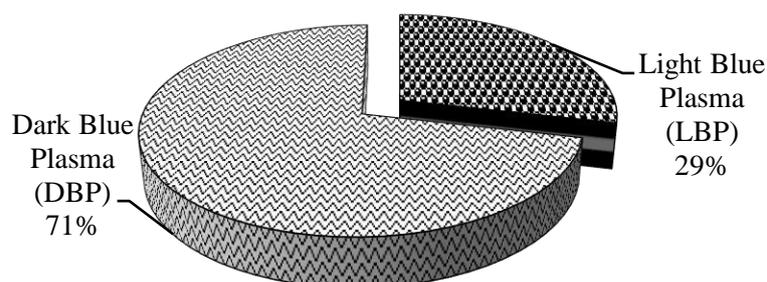


Figure 4. Plasma component proportion of horseshoe crab (%) from the study site

3.2.2. Bacterial inhibition activity of extracted plasma biomass of *Carsinoscorpius rotundicauda*

The sample fraction of Dark Blue Plasma (BDP) and Light Blue Plasma (LBP) from *Carsinoscorpius rotundicauda* around the Madura Strait have a higher inhibition zone against gram positive bacteria than gram negative bacteria. The total average of plasma inhibitory activity of this organism in gram-positive bacteria; *Staphylococcus aureus* 10.00 mm and *Bacillus* 10.07 mm for DBP while that *Staphylococcus aureus* 9.11 mm and *Bacillus* 9.67 mm for LBP samples (Table 1). The same potential is also present in extracted carapace of *Carsinoscorpius rotundicauda* using acetone palate which is capable of inhibiting *Bacillus* bacteria by 6 mm/30 μ l and *Staphylococcus aureus* bacteria 7 mm/30 μ l [16].

One of the factors affecting the high activity of inhibition zone of two plasma fraction of horseshoe crab against gram-positive bacteria is wall structure of the bacterial cell and the existence of bacteria on the body organ of horseshoe crab. *Staphylococcus aureus* bacteria have a single-cell-layer with relatively simple structure thereby allowing the entry of substances potentially damage bacterial cells [34]. *Bacillus* is commonly found in the dorsal and ventral portions of horseshoe crab larvae [35], so plasma horseshoe crab is used as a material to protect their body from bacterial infection. *Bacillus* infections may cause changes in egg color (red, gray, black), interfere the egg's cells growth, disrupt the movement of larvae and cause high mortality after molting [35]. This bacterial infection can also cause stress on horseshoe crab by interrupting mobility and slowing down blood clotting reaction of horseshoe crab [36].

Inhibitory activity of extracted horseshoe crab plasma biomass on bacteria in each sample also showed variation (Table 1). Extracts of DBP and LBP of *Carsinoscorpius rotundicauda* formed the highest inhibition zone on gram-positive bacteria are samples from Bangkalan waters. The lowest inhibition zone of the DBP sample is found in horseshoe crab from Sumenep waters, while the lowest inhibition zone of LBP is present in horseshoe crab from Probolinggo waters. The inhibition zone of DBP from extracted horseshoe crab samples of Bangkalan waters against *Staphylococcus aureus* bacteria was 10.67 mm while that *Bacillus* was 10.89 mm. The LBP samples resulted in inhibition zone of 9.33 mm for *Staphylococcus aureus* and 10.00 mm for *Bacillus*.

Table 1. Average inhibition of biomass from *Carsinoscorpius rotundicauda* plasma extract on test bacteria

Station	Average Zone of Inhibition (mm)			
	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>	<i>Vibrio harveyi</i>	<i>Bacillus</i>
Dark blue plasma (DBP) sample				
Sumenep	8.00	10.00	9.67	10.00
Bangkalan	-	10.67	-	10.89
Probolinggo	9.00	9.33	9.00	9.33
Total	8.50	10.00	9.33	10.07
Light blue plasma (LBP) sample				
Sumenep	7.33	9.67	9.00	9.67
Bangkalan	-	9.33	-	10.00
Probolinggo	7.33	8.33	7.67	9.33
Total	7.33	9.11	8.33	9.67
Control	15.00	20.00	14.67	27.67

The high capability of DBP and LBP horseshoe crab from Bangkalan waters to inhibit bacteria is suspected due to the horseshoe crab living in this location has high environmental stress compared to the horseshoe crab from Probolinggo and Sumenep waters. The high capacity of the samples from this location to inhibit bacterial growth is an indication of self-defense efforts of horseshoe crab against gram-positive bacteria in the environment. Horseshoe crab will produce anti endotoxin and anti-bacterial compounds within their body's immune system by conducting an intracellular freezing system to defend against pathogenic organisms [37]. Plasma *Carsinoscorpius rotundicauda* produces *Carsinoscorpius* Amoebocyte Lysat (CAL) or called *carsinoscorpin*, a compound in horseshoe crab blood that acts as a growth inhibitor or bacterial killer and detects endotoxins in horseshoe crab [38]. The pure extract of this compound is capable of tolerating and maintaining the life of *Carsinoscorpius rotundicauda* from high concentrations of *Escherichia coli* bacterial infections of 10^6 CFU/ml in laboratory scale [39]. This shows that the production of immunity horseshoe crab will increase along with the environmental stress conditions, one of which is to increase the inhibition of bacteria in their body.

Plasma of *Carsinoscorpius rotundicauda* has a moderate inhibitory ability against *Escherichia coli* and *Vibrio harveyi*. This is evidenced by the small diameter of inhibition zones of these two bacterial in BDP and LBP of horseshoe crab samples from Sumenep and Probolinggo waters. Based on the clear

zone diameter, the capability of bacterial inhibition is classified into 3 groups, that is 10-20 mm as strong inhibitory power, 5-10 mm as moderate inhibition power and <5 mm as low inhibitory power [40]. The low inhibition zone of horseshoe crab from from the waters of Madura on *Escherichia coli* and *Vibrio harveyi* is thought to be caused by layers of bacterial cell wall structures. *Escherichia coli* has a three-layered cell wall structure composed of lipopolysaccharides, peptidoglycan, and proteins that cause difficult entry of cell destructive substances [40]. In contrast to *Escherichia coli*, *Vibrio* sp is a bacterium associated with horseshoe crab within a metabolic system and has a mutualistic symbiotic relationship in producing bioactive compounds [41].

3.3. Correlation between Biomaterial Compound and Bioactivity of Plasma

Production of the biomaterial compound of horseshoe crab is closely related to the potency of horseshoe crab bioactivity to inhibit bacteria. Several factors affect the size of the inhibition zone are biomaterial compounds, the types of biomaterial compounds, the nature of bacterial resistance to the biomaterial substance levels, and bacterial density [42]. The result of analysis of the principal components on correlation between the biomaterial compound and inhibitory activity of light blue plasma of horseshoe crab was formed by two main axes, F1 with the variance of 50.85% and F2 of 20.05% (Figure 5).

The same results were also obtained on dark blue plasma horseshoe crab samples. Analysis of the principal components of the biomaterial compound and the inhibitory activity of the dark blue plasma from horseshoe crab sample were formed by two major axes namely F1 and F2 with a total range of 86.75% (Figure 6). Based on the results obtained it is known that the high content of zoosterol of horseshoe crab from Bangkalan waters (ST2U1, ST2U2, ST2U3) is parallel to the ability of DBP sample to inhibit *Bacillus* and *Staphylococcus aureus*. This corresponds to the close correlation value of the zoosterol compound to *Bacillus* (0.768) and *Staphylococcus aureus* (0.734) (Table 2). The high level of zoosterol compounds in horseshoe crab meat can inhibit gram-positive bacteria higher than the gram-negative bacteria. The inhibitory power of DBP against gram-negative bacteria is formed on the negative axis F1 on ST1 and ST3, indicates that the inhibitory of the sample on *Escherichia coli* and *Vibrio harveyi* is highest in Sumenep and Probolinggo waters. The width of the inhibitory zone diameter indicates the stronger inhibition of biomaterial compounds on bacterial growth [43]. Steroid compounds play a role in inhibition of bacterial activity by destroying bacterial cell membranes [44]. The ability of these compounds to penetrate cell membrane layers can lead to leakage and damage of bacterial cells [45]. The three most important types of zoosterol are cholesterol, steroid hormone, and coprostanol [46]. Cholesterol in the blood circulates in lipoprotein particles which are complex compounds between fat and protein [47]. Horseshoe crab from *Tachypleus tridentatus* plasma contains tachycitin [48], a type of protein that has a molecular weight of 380-440 kDa that is potential as an antibacterial. Some amino acid derivatives sterols (zoosterol) in cholesterol synthesized from animal bile acids have the ability to inhibit the strong growth of *Staphylococcus aureus* bacteria with a minimum concentration of 6.25 MIC inhibition, but require considerable concentrations to inhibit *Escherichia coli* >50 MIC [49].

Table 2. The correlation of samples and test bacteria on the principal components of F1 and F2

Variable Bacteria	<i>Dark blue plasma sample</i>		<i>Light blue plasma sample</i>	
	Monoterpenoid	Zoosterol	Monoterpenoid	Zoosterol
<i>Escherichia coli</i>	-0.3736	-0.6656	-0.3270	-0.6549
<i>Staphylococcus aureus</i>	0.4554	0.7336	0.1845	0.0880
<i>Vibrio harveyi</i>	-0.3541	-0.6111	-0.4160	-0.6369
<i>Bacillus</i>	0.3368	0.7680	-0.1534	0.3747

The high inhibition zone of LBP and DBP of *Carsinoscorpius rotundicauda* on bacteria from Bangkalan waters is supported by the high content of biomaterial compounds produced by horseshoe crab. The conditions of Bangkalan waters is suspected to be the cause of horseshoe crab living in this location produce higher secondary metabolite compounds in the form of monoterpenoid and zoosterol to adapt and defend themselves from environmental stresses, one of which is antibacterial.

Environmental conditions of the organism are able to stimulate secondary metabolic processes as a response to increase self-defense activities [50]. The response form is carried out chemically by producing secondary metabolite compounds as an adaptation material to its environmental conditions [51], defense materials from other organism attacks [20] eg predators, parasites and microorganisms such as bacteria forming biofilms, fungi, epiphytic algae, and invertebrates [52]. Bioactivity of extract from organism against bacteria is influenced by environment condition of the waters. Organisms living in polluted aquatic environments tend to have higher bioactivity than that organisms in relatively clean waters. The high bioactivity of organism in polluted waters since these organisms produce a lot of secondary metabolite as a survival substance for infection prevention of pathogenic microorganism [53].

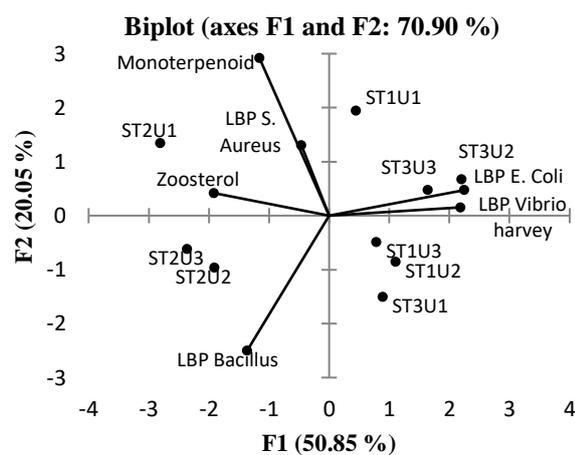


Figure 5. Principal Component Analysis of biomaterial content and bacterial inhibition of LBP sample of horseshoe crab.

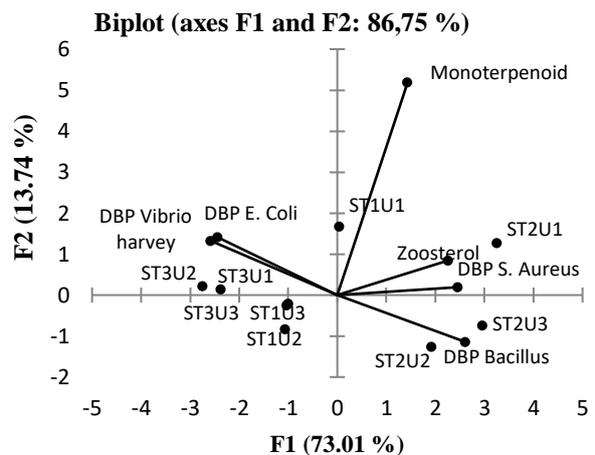


Figure 6. Principal Component Analysis of biomaterial content and bacterial inhibition of DBP sample of horseshoe crab.

4. Conclusions

The highest content of monoterpenoid and zoosterol in *Carsinoscorpius rotundicauda* biomass is obtained by horseshoe crab living in Bangkalan waters, while the smallest of three locations found in horseshoe crab living in Probolinggo waters. Bioactivity of sample from both extracted plasma produced the highest bacterial inhibition zone in *Staphylococcus aureus* and *Bacillus*, which is samples from Bangkalan waters, while that the highest *Escherichia coli* and *Vibrio harveyi* inhibition zones was found from Sumenep waters. The lowest gram positive and gram negative bacterial inhibitor zone is found in Probolinggo waters.

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