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Potential of Sea Cucumber Rivet Red Extract (Holothuria leucospilota) As Antibacterial MDR (Multi Drug Resistant)

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ABSTRACT

Resistance of microorganisms to antibiotics is a serious problem in the treatment of infections of this period, so that necessity search for new bioactive compounds was inevitable. Secondary metabolites found in marine organisms is one of the alternative materials discovery of new antibiotics. The potential producers of bioactive compounds of secondary metabolite is sea cucumber. Rivet sea cucumbers *Holothuria leucospilota* is one of sea cucumbers found in abundance in Bandengan coast Jepara, but so far very limited use for marine pharmacology. The purpose of the study was to determine the antibacterial activity of the extract fractions rivet red sea cucumber (*H. leucospilota*) against MDR bacteria and compounds that have potential as antibacterial MDR. Analysis of samples of sea cucumbers includes extraction, fractionation, and analysis of bacterial sensitivity test Gas Chromatography-Mass Spectrometer (GC-MS), the extraction process is carried out by solid-liquid extraction method (solid-liquid). Fractionation is done with Open Column Chromatography (OCC). Test sensitivity of bacteria using the agar diffusion method according to the Kirby-Bauer. The results showed that the extract of Holothuria leucospilota has 7 fractions active against MDR bacteria *Klebsiella* sp and *Enterobacter-10*. The average value of inhibition zone is highest in fraction III with concentration of 80 ug / disk for each type of bacteria *Klebsiella* sp and *Enterobacter* 10 was 11.87 ± 0.90 mm and 11.49 ± 0.86 mm. The results of GC-MS analysis showed that the fraction IV containing the compound 2-methyl butanoic; 2-butoxy ethanol; 3,5,5 trimethyl 2-cyclohexane-1; propandiat phenyl and 2-methoxy-4-(2-propenyl) or eugenol.

Keywords: *Holothuria leucospilota, extract, MDR bacteria, antibacterial*

INTRODUCTION

MDR (*Multi Drug Resistant*) bacteria has defined as bacteria with resistant to a group of antibiotic. This was since the resistance as a natural mechanism for bacteria to survive antibiotic. Therefore there must be an important evort in finding and explore new biosubstances for new MDR bacteria. So far antibiotic explorations rely on the terrestrial organism, while actually Indonesia known as the rich marine organism with high potential for marine pharmacology. Indonesia marine organism with its geographical position had developed a unique environment with high marine biodiversity with high potency of secondary metabolites to be devoped for human health. A group of marine organism with high potency to be developed for secondary metabolites was marine invertebrate. These marine invertabrates has a very limited physical movement compared with other marine vertebrates. So that they developed a good defence system with producing many biosubstances. More specifically these biosusbstances or secondary metabolites were used for self protection especially from microbial infections with assumption that their secondary metabolites have highly prospective as an active biosusbstances against bacterial infections, neurology, anti-inflammatory, antivirus, and anticancer. One class of marine invertebrate which produce secondary metabolites is sea cucumber (Holothuroidea).

Sea cucumber has a secondary metabolite known as holothurin. Holothurin was widely used in pharmacological as antifungal treatment and anticancer (Bhakuni and Rawat, 2006), antibacterial and antitumor (Mojica *et.al.*, 2007). Utilization of appropriate antibiotics will lead to resistant bacteria, it is

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necessary to search new antibiotic compounds are more effective and efficient in addressing the issue of bacteria Multi Drug Resistant (MDR). Various approaches have been made to obtain new antibiotic compounds, one of which is the search for bioactive compounds from microorganisms that are symbiotic with marine invertebrates, the molluscs (Pringgenies, 2010a) and potential as an anti-bacterial Multi Drug Resistant (MDR) (Pringgenies, 2010b). Zhu et al. (2007) has been successfully screened a number of 42 isolates of bacteria, these bacteria isolated from organisms, water and marine sediments as well as the potential to produce antibacterial compounds. Pringgenies (2009) have isolated more than 20 types of gastropods and show that there are about 10 species of gastropods that have a very promising antibacterial activity to be used as new material on antibiotic. The aim of the research was explore the antibacterial activity from extract of *Holothuria leucospilota* and the bioactive substances for MDR bacteria.

MATERIALS AND METHODS

Materials. Main material in the research were 50 samples of *H. leucospilota* collected from Bandengan coastal waters, Jepara. Test bacteria used were *Enterobacter-10*, *Enterobacter*-5, *Escherichia coli, Pseudomonas* sp., *Klebsiella* sp. and *Coagulase Negative Staphylococcus* supplied by Mikrobiology Laboratory of Dr. Kariadi hospital, Semarang.

Preparation and sample ekstraxction. *H. leucospilota* blackish-brown colour sea cucumber and will actively excretes white glutinoeous sap (gum) from the mouth. Sample of sea cucumbers were cleaned and rinsed thoroughly on running water, dissected the stomach to excludes the stomach contents and the wet weight arround 2327.35 g. Sample of *H. leucospilota* then dried oven with temperature of 45 ^{o}C for 48 hours. Dried samples with arround 244.39 g weight then sliced with about 0.5 cm thickness for exctraction. Exctraction was done using a stepwise solid-solution methode. Etraction was done by soaking 244.39 gram of dry sample in volume 900 ml n-heksan solution for 24 hour, and later the solvent was filtered. The residue was then soak again in the same solvent for 2 hours for several times until solvent becomes clear. Filtrates then soaked in etil-acetate and methanol as in n-heksan methode. Filtrate was then evaporated using rotavapor at temperature of 40 ^{o}C (Mojica *et.al.*, 2007).

Weight of exctract was calculated using formula :

$$We = Wv_2 - Wv_1$$

Where : We = exctract weight

Wv_1 = weight of empty vial

Wv_2 = eight of vial and exctrat

Percent exctract content :

$$Ce = \frac{W_2}{W_1} x100\%$$

Where : Ce = percent of exctract content in the sample

W_1 = sample weight

W_2 = exctract weight

Positive and negative control test to tested bacteria.

Positive control test. Positive control test was done using antibiotic streptomycin sulfate and amoxicilin which were presence in the market with concentration of 20 µg/disc. These test aimed to show the resistance zone performed by antibiotic, so that can be comparred with antibacterial performance by exctract of *H. leucospilota*.

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Negative control test. Negative control test was done using three solvent previously used in the exctraction processes, that are n-heksan, etil acetate and methanol to the tested bacteria. This was to checked whether there are any effect of the solvent to the perform of resistance zone by the exctract.

***H. leucospilota* exctract test to the tested bacteria (MDR).** *H. leucospilota* exctract test to MDR bacteria was done n-heksan, etil acetate, and methanol exctract. Concentration used were 80 µg/disc, 40 µg/disc, 20 µg/disc, 10 µg/disc and 5 µg/disc (Nagarajappa and Goswami, 2007). A paper disc was laid down on the plate agar already contain with the MDR bacteria. Then 10 µL of *H. leucospilota* exctract was dropped onto the paper disc with concentration of 8 µg/µL, 4 µg/µL, 2 µg/µL, 1 µg/µL and 0,5 µg/µL. Observtion of the resistance zone after 24 hour.

Thin Layer Chromatography (TLC). TLC analysis on the etil acetate *H. leucospilota* exctract was done using stable phase of silica gel F_{254} with several combination as a moving fraction. The TLC formed was then sliced with 5 cm length and 1 cm width (Gandjar and Rohman, 2007). At every TLC end a 0,5 cm line from the start to the end TLC. Five percent concentration of the exctract was then gently touched down onto the middle of the start line of the TLC using a capillary syringe. The TLC with addition of exctract was then put into a beaker glass with combination of the three solvent (methanol, etil acetate and n-heksan). Beaker glass was closed tightly until efluent goes to final end, the TLC plate was lifted and dried. Formed spot was observed using UV light (Sthal, 1985) and note the Rf value. Rf value was define as follows (Yazid, 2005):

$$Rf = \frac{\text{Distance of spot from start lines}}{\text{Distance of the solvent}}$$

Open Column Chromatography (OCC). OCC analysis was aimed to separate fraction of biosubstances in the exctract based on its polarity levels (Kristanti dan Aminah, 2008). Etile acetate *H. leucospilota* 0.4 g was fractionated using 60-silica gel OCC (0.2 – 0.5 mm, Merck) weight 12 gram as solid phase. Etil acetate and chloroform were used with ratio of 3:1. Column used was firstly cleaned with solid and flat cotton and solvent at the base of the column to avoid any air buble and a layer of paper disc on top. Silica gel 12 g was firstly activated in the oven with 120 ^{o}C emperature for 1 hour. Then 10 gram of it was mixed with the solvent for 2 hours, then put into the column solid anf flat to avoid air bubles. On top of the silica gel covered with filter paper and let to form solid plate for 24 hour. Etil acetate *H. leucospilota* exctract weight of 0.4 g was diluted in the solvent then add 2 g of silica gel, mixed with homogenously and keep until solvent had completely evaporated and put into the column which already preparated for 24 hours. Open the column valve with flow of 1 drop/second and countinuously add solvent into the column, where silica gel should kept in soaking with the solvent. Efluante from the column was collected in a vial with volume of 5 mL for analysis using TLC. Similar spot patern of the column was put together for evaporation.

***H. leucospilota* Exctract Fraction Activity Test for the MDR Bacteria.** Activity test was done with diffusion methode or disc methode of Kirby-Bauer (Lay, 1994). Each fraction concentration were 80 µg/disc, 40 µg/disc and 20 µg/disc. Antibiotic concentration used was 20 µg/disc (Appendix-1). Tested bacteria was firstly inoculated in a *Nutrient Broth* /NB (Appendix-2) and incubated for 24 hours. Abundance of tested bacteria was 0.5 as in *Mc Farland* (Nakamura *et.al.*, 1999) and keep for 5 minutes

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(Lay, 1994). Paper disc was laind down on the agar medium with tested bacteria and then 10 µL exctract fraction of etil acetate *H. leucospilota* slowly dropped onto the paper disc with cconcentration of 8 µg/µL, 4 µg/µL and 2 µg/µL (Appendix-1). Observation on the resistance zone was done every 24 hours for three days. Activity test was done for three times.

Gas Chromatography- Mass Spectrometry (GC-MS). GC-MS analysis was done for IV fraction with 0.1 ml volume injection. Column used was Rtx-5Ms with 30 meters length and strat temperature of 80 ^{o}C. Capilar diameter was 0.25 mm. Exctract samples injected into the injektor with end temperature of 320 ^{o}C and speed of 10 ^{o}C/minute and will directly evaporated and would be associated with helium gas with speed of 27.3 cm/sec.

RESULTS AND DISCUSSIONS

Sample exctraction. Exctraction result on 244.39 g dry weight of *H. leucospilota* using three solvents n-heksan, etil acetate and methanol were as presented Table 1. Exctration result using three different solvent had gave different exctract weight and percentage. Where methanol solvent had produce the highest exctrat dry weight of 34.14 g and exctract percentage of 3.97%. While etil acetate solvent gave the smallest exctract that is 2.24 g and exctrat percentage of 0.92%.

Table 1. Exctraction result of *H. leucospilota* using three solvent

Solvent	Dry weight (gram)	Exctract percentage	Form	Colour	Odor
n-Heksan	4.78	1.96	Paste	Dark brown	Fishy
Etil acetate	2.24	0.92	Oil	Light brown	Fishy
Methanol	34.14	13.97	Paste	Greenish brown	Fishy

Positive and negative control test to MDR bacteria

Positive control test. Result of positive control test had showed that antibiotic of *Streptomicyn sulfate* can resist all of tested bacteria, in contrast *Amoxicilin* fail to resist all of tested bacteria. *Streptomicyn sulfate* had formed the biggest resistance zone to *Klebsiella* sp. That is 8.89 mm and smallest to *Coagulase Negative Staphylococcus* that is 7.24 mm. The complete result test was presented as in Table 2.

Negative control test. Negative control test of the three solvent had no effect on the tested bacteria, where no resistance zone shown on the paper disc as presented in Table 3.

Table 2. Result of Positive Control Test to MDR Bacteria

Tested bacteria	Diameter of resistance zone (mm)	
	Streptomicyn sulfate	*Amoxicylin*
Enterobacter 10	8.72	0
Enterobacter 5	7.99	0
Escherichia coli	8.40	0
Coagulase Negative Staphylococcus	7.24	0
Pseudomonas sp	8.10	0
Klebsiella sp	8.89	0

Table 3. Result of Negative Control Test to MDR Bacteria

Tested bacteria	Diameter of resistance zone (mm)		
	n-Heksan	Etil acetate	Methanol
Enterobacter 10	0	0	0
Enterobacter 5	0	0	0

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Escherichia coli	0	0	0
Coagulase Negative Staphylococcus	0	0	0
Pseudomonas sp.	0	0	0
Klebsiella sp.	0	0	0

Activity test of *H. leucospilota* exctract to MDR bacteria. Activity test of *H. leucospilota* exctract with three solvents to tested bacteria had resulted that n-heksan solvent give no resistance zone to all tested bacteria. Exctract with etil acetate had performed a resistance zone to 4 tested bacteria *Enterobacter*-10, *Klebsiella* sp., *Pseudomonas* sp. and *Enterobacter*-5. Exctrat with methanol had performed the resistance zone to two tested bacteria *Pseudomonas* sp. and *Klebsiella* sp. All in complete as presented in Table 4. MDR bacateria *Klebsiella* sp. and *Enterobacter*-10 which were the most resisted by exctrat of *H. leucospilota* later used for activity test of exctract fraction. This result was proved by larger diameter formed and the clarity of the resistance zone.

Table 4. Result of Activity Test of *H. leucospilota* Exctract to MDR Bacteria

Tested bacteria	Diameter of resistance zone (mm)		
	n-Heksan	Etil acetate	Methanol
Enterobacter 10	0	9.75	0
Enterobacter 5	0	9.65	0
Escherichia coli	0	0	0
Coagulase Negative Staphylococcus	0	0	0
Pseudomonas sp.	0	9.32	9.21
Klebsiella sp.	0	9.68	7.01

Result of Thin Layer Chromatography (TLC). Thin Layer Chromatography (TLC) analysis was done for optimum search of the specific solvent that can separate exctract component. This was done by means of solvent ratio combination which had produce different number of spots. Each spot had a different Rf value as presented in Table 5. Based on the result as in Table 5 shows that optimum solvent combination in fractionated exctract components was etil acetate : chloroform with ratio of 3 : 1 which had produce 3 spots with Rf value of 0.64; 0.73 and 0.84. Then this solvent combination and ratio was used as solvent for Open Chromatography Column.

Table 5. Fractionation Test of Exctract Component of *H. leucospilota* by Solvent

No.	Solvent combinations	Ratio	No of spot	Rf value
1	Etil acetate	1 : 0	1	0.92
2	Etil acetate : methanol	1 : 1	1	0.9
3	Etil acetate: methanol	1 : 2	1	0.59
4	Etil acetate: n-Heksan	1 : 1	1	0.81
5	Etil acetate: n-Heksan	2 : 1	1	0.73
6	Etil acetate: chloroform	1 : 1	2	0.57; 0.7
7	Etil acetate: chloroform	2 : 1	2	0.59; 0.73
8	Etil acetate: chloroform	3 : 1	3	0.64; 0.73; 0.84

Open Chromatography Column (OCC). Open Chromatography Column analysis using 30 vials with 5 ml volume each and grouped into 7 fractions. Complete result of OCC was presented as in Table 6 which shows that the biggest weight of fraction was given by fraction IV with 0.1204 g and fraction I produce the smallest fraction with 0.0156 g. Each fraction had shown different number of spots, with the highest number of spot was given by fraction IV.

Tabel 6. Result on Groups of OCC Efluent

No. vial	Weight (gram)	Spot number	Rf value	Fraction

1	0.0156	1	0.93	I
2	0.0544	1	0.99	II
3	0.0712	2	0.48; 0.97	III
4-5	0.1204	**4**	0.23; 0.72; 0.84; 0.9	**IV**
7-8	0.0249	3	0.68; 0.72; 0.76	V
9-10	0.0164	2	0.68; 0.76	VI
11-30	0.0625	2	0.68; 0.84	VII

Activity Test of Fraction by OCC to MDR Bacteria. Exctract fraction yielde from OCC analysis were tested again for the antibacterial activity differenciate the most active fraction to *Klebsiella* sp. and *Enterobacter*-10. The result of activity test of fraction I-VII to *Klebsiella* sp. was in general shows that addition of fraction concentration had effected on the diameter of resistance zone, the highest concentration the more size of the resistance zone. Result of activity test of fraction to *Klebsiella* sp. was presented as in Table 7.

Table 7. Result of Fraction I-VII Activity Test to *Klebsiella* sp.

Concentration (µg/disc)	Fraction	Diameter of resistance zone (mm)		
		24 hour	48 hour	72 hour
20	I	8.43 ± 0.36	8.35 ± 0.34	8.72 ± 0.91
	II	8.74 ± 0.83	9.38 ± 0.86	8.88 ± 0.84
	III	9.74 ± 0.45	9.48 ± 0.73	9.51 ± 0.75
	IV	9.71 ± 0.51	9.95 ± 0.98	9.50 ± 0.85
	V	8.60 ± 0.70	8.75 ± 0.62	8.41 ± 0.55
	VI	9.39 ± 0.66	9.54 ± 0.23	9.07 ± 0.10
	VII	8.41 ± 0.66	8.95 ± 0.50	8.49 ± 0.71
40	I	11.68 ± 0.33	11.52 ± 0.38	10.82 ± 0.83
	II	9.56 ± 0.64	9.1 ± 0.65	8.95 ± 0.94
	III	9.73 ± 0.35	9.36 ± 0.85	9.44 ± 0.76
	IV	10.04 ± 0.19	9.87 ± 0.68	9.79 ± 0.32
	V	9.69 ± 0.85	9.47 ± 0.93	9.26 ± 0.76
	VI	9.86 ± 0.06	9.51 ± 0.45	9.38 ± 0.46
	VII	8.57 ± 0.29	8.6 ± 0.66	8.19 ± 0.47
80	I	10.32 ± 0.84	10.34 ± 0.42	9.89 ± 0.45
	II	9.82 ± 0.95	9.32 ± 0.56	9.16 ± 0.55
	III	11.44 ± 0.45	11.63 ± 0.76	11.87 ± 0.90
	IV	10.65 ± 0.79	10.63 ± 0.66	10.25 ± 0.34
	V	10.69 ± 0.51	10.07 ± 0.23	10.09 ± 1.00
	VI	9.66 ± 0.20	9.38 ± 0.24	9.15 ± 0.39
	VII	9.26 ± 0.23	8.93 ± 0.72	8.51 ± 0.79

- Mean ± SD
- SD = Standard Deviation

Table 8. Result of Fraction I-VII Activity Test to *Enterobacter*-10

Concentration (µg/disc)	Fraction	Diameter of resistance zone (mm)		
		24 hours	48 hours	72 hours
20	I	9.54 ± 0.88	9.29 ± 0.33	8.94 ± 0.74
	II	10.06 ± 0.41	8.84 ± 0.48	9.24 ± 0.59
	III	9.89 ± 0.36	9.79 ± 0.25	9.26 ± 0.08
	IV	10.75 ± 0.82	9.89 ± 0.26	9.85 ± 0.58
	V	9.13 ± 0.31	9.23 ± 0.86	9.13 ± 0.51

	VI	9.91 ± 0.28	10.15 ± 0.49	9.27 ± 0.14
	VII	9.94 ± 0.74	9.55 ± 0.67	9.03 ± 0.65
40	I	9.64 ± 0.49	9.93 ± 0.56	9.95 ± 0.44
	II	8.77 ± 0.84	9.29 ± 0.59	9.15 ± 0.65
	III	9.79 ± 0.06	9.21 ± 0.76	9.19 ± 0.59
	IV	9.72 ± 0.31	9.63 ± 0.76	9.29 ± 0.19
	V	9.61 ± 0.19	10.19 ± 0.68	10.19 ± 0.61
	VI	9.15 ± 0.64	9.29 ± 0.36	8.99 ± 0.55
	VII	8.51 ± 0.40	8.48 ± 0.27	8.47 ± 0.77
80	I	9.43 ± 0.18	8.48 ± 0.52	8.82 ± 0.38
	II	9.83 ± 0.86	9.20 ± 0.64	9.17 ± 0.96
	III	11.49 ± 0.86	10.88 ± 0.16	10.87 ± 0.89
	IV	9.97 ± 0.81	9.36 ± 0.88	9.37 ± 0.47
	V	9.74 ± 0.48	9.12 ± 0.41	9.15 ± 0.71
	VI	10.11 ± 0.26	8.94 ± 0.47	9.25 ± 0.73
	VII	8.36 ± 0.41	7.88 ± 0.36	7.92 ± 0.41

- Mean ± SD
- SD = Standard Deviation

While in contrast result of fraction test of I-VII fraction to *Enterobacter* sp was in general shows that higher concentration of fraction had no effect to the diameter size of the resistance zone. In general concentration of 40 µg/disc fraction I-VII had shown the resistance zone and result of fraction test to *Enterobacter*-10 was presented as in Table 8.

Gas Chromatography-Mass Spectrometry (GC-MS). Analysis using GC-MS to fraction IV had shows 6 intensity peaks, means that this fraction have 6 bioactive substances as in Figure 1 and result of bioactive compound identification of this fraction had been presented as in Table 9.

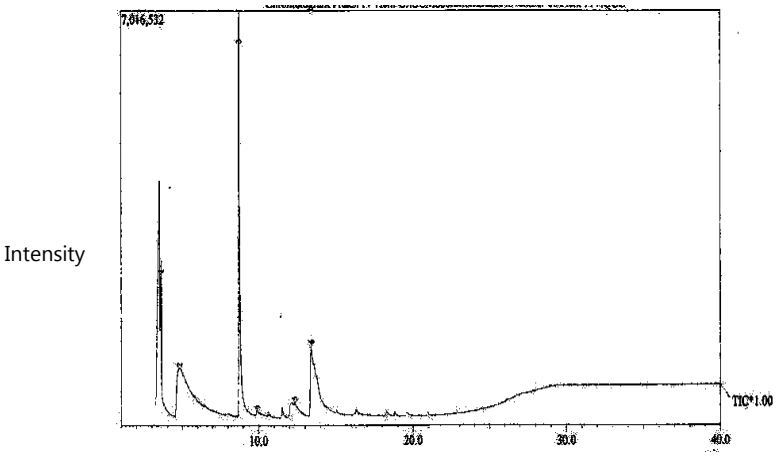

Figure 1. Result of GC-MS analysis with 6 intensity peaks

Table 9. Result of GC-MS Analysis on Fraction IV

No.of peak	Retention time	Area (%)	SI (Similarity Index)	Name of bioactive compound
1	3.620	27.53	95	2-methil butanoat
2	4.825	24.01	98	2-butoxi ethanol
3	8.751	22.03	97	3,5,5 trimetil 2-sicloheksan-1
4	9.909	1.93	-	unidentified
5	12.359	4.82	87	Phenil propanadioat
6	13.404	19.68	91	2-metoxi-4-(2-prophenil)/ eugenol

DISCUSSIONS

Positive control test had shown that there were some differences between Streptomicyn sulfate and Amoxicilin in resisting to MDR bacteria. Streptomicyn sulfate have capability in chalenging all of tested bacteria, while amoxicilin did not have such capability. This result was means that the tested bacteria (MDR bacteria) had already resistance to Amoxicilin. According to *National Committee For Clinical Laboratory Standard* (NCCLS), resistance zone diameter less than 11 mm for *Streptomicyn sulfate* (10 µg/disc) was categorized as resist and *Amoxicilin* (10 µg/disc) resistance zone diameter less than 13 mm. Negative control test which had shown that the three solvent used did not show a resistance zone to tested bacteria. While application of *H. leucospilota* exctract had significantly give antibacterial activity the formation of resistance zone on the paper disc, meaning that the resistance zone was really caused by exctrat of *H. leucospilota*. Trianto *et.al*. (2004) that the presence of the resistance zone in the paper disc soaking in the exctrat had proved that the bioactive compound in the exctract had effectively works and not because the effect of the solvent.

Lay (1994) stated that chalenges activity to the grow of bacteria by antibiotics wold be shown by a clear zone suroundings of the paper disc. In more detail the research had also revealed that etil acetate exctract of *H. leucospilota* performed an active antibacterial activity to *Enterobacter*-10, *Klebsiella* sp., *Pseudomanas* sp., and *Enterobacter*-5. Methanol exctract of *H. leucospilota* active to *Pseudomanas* sp. and *Klebsiella* sp., and n-heksan *H. leucospilota* exctract show no antibacterial activity to all of tested bacteria. In other perspective these findings had confirmed that non polar biosubstances in the exctract of *H. leucospilota* had no antibacterial capability to all tested bacteria. Differences on the antibacterial activity of the ectract was presumably due to polarity of the exctract. According to Hartini *et.al*. (2008) that polarity of a biosubstance that will effect the penetration of the cell wall of the bacteria. Biosubstance with polarity will get easier in penetrating the cell wall. Purwoko (1998) mention that a semi-polar to non-polar biosusbtances have more potential to form a toxin effect, which is difficult to be excreted by organism comparred to polar biosubstances.

Based on TLC analysis that solvent which can produce the best fractionation on the exctrat of etil acetate *H. leucospilota* was combination of etil acetate : chloroform with ratio of 3 : 1, which can form 3 spots as in Table 5. Spot formed on a silica plate could not be seen directly, but should use UV. Kristanti and Aminah (2008) mention that the use of UV light was one methode to observe a spot on silica plate. Stahl (1985) explain that the most simple detection methode is when the biosubstance shows an absortion in the short wave UV light (256 nm) or long wave length (365 nm).

Capability of solvent in biocompound fractionation in exctract was shown by the formation of spot with different peaks. Which later the combination of solvent used for OCC analysis. As already stated earlier that etil acetate axctract of *H. leucospilota* had a different polarity, that is based on the different Rf

value. According to Sastrohamidjodjo (2001), that every compound with different Rf value on two peaks on OCC indicates of two different biocompounds. A compound with Rf value aproaching to null was indicated as a polar compound. While a compound with Rf value near to one was diffined as a non-polar compound. TLC and OCC analysis which were categorized into 7 groups fraction IV had yielded the weight of 0.1204 g which etil acetate exctract of *H. leucospilota* had confirmed with more biosubstances content.

Antibacterial activity test of etil acetate fraction I – VII of *H. leucospilota* exctract to *Klebsiella* sp. and *Enterobacter*-10 had performed a larger diameter and clear resistance zone comparred to other bacteria *Enterobacter*-5, *Escherichia coli*, *Coagulase Negative Staphylococcus* and *Pseudomonas* sp. Activity test of fraction I – VII using etil acetate of *H. leucospilota* to *Klebsiella* sp. and *Enterobacter*-10 shows that fundamentally all fraction have a capability as antibacterial agent. This was shown with the formation of resistance zone at surrounding of the paper disc as also stated by Lay (1994). Fration I – VII exctract using etil acetate *H. leucospilota* forming a larger diameter of the resistance zone if compared using *Streptomicyn sulfate* or *Amoxicilin* with the same concentration of 20µg/disc, meaning that the fraction exctrat of *H. leucospilota* had a better antibacterial activity. Result of antibacterial activity of the exctrat fraction to *Klebsiella* sp had shown diffrerences in the formation of the resistance zone diameter of each fraction. Seven fractions with different concentration 20 µg/disc, 40 µg/disc, 80 µg/disc had shown a different result during incubation. Fraction III with concentration of 80 µg/disc had formed the largest diameter of resistance zone, means that this fraction is the most effective in challenging the growth of *Klebsiella* sp. Increasing concentration of fraction II, III, IV, V and VII to *Klebsiella* sp. which will increase the diameter of the resistance zone. This was assumed that increase of exctract concentration will also increase bioactive compound in the exctrat, as also mentioned by Prijono (1994). Fraction I and VI will already increase the resistance zone diameter at concentration of 40µg/disc, mean as this fraction have effectiveness in challenging the growth of *Klebsiella* sp.

Result of activity test of the seven fractions with different concentration 20 µg/disc, 40 µg/disc, 80 µg/disc to *Enterobacter*-10 have a diffent activity during incubation. Fraction III with concentration of 80 µg/disc was the most effective in resisting the growth of *Enterobacter*-10 with larger resistance zone diameters. Increasing of concentration of the seven fraction have no effect on the diameter of the resistance zone. Fraction IV, VI and VII have already resisting the growth of bacteria at concentration of 20 µg/disc. While fraction I and V start resisting at concentration of 40 µg/disc and fraction II and III at concentration of 80 µg/disc.

In general the seven fraction had functioned as bacteriostatic capability and in 48 hours incubation had already dreseasing the resistance zone diameter. According to Wattimena *et al.* (1991) an antibacterial compound classified as bacteriostatic when exhibiting a narrow resistance zone and decrease of the zone clarity after 24 hours incubation, but will be classified as bactericide if still shows clear resistance zone after 48 hours incubation.

The seven fraction result from OCC had shown different capability in challenging the growth of *Klebsiella* sp. and *Enterobacter*-10, based on the diffent diameter of the resistance zone. The differences of the resistance zone diameter were caused by the difference of bioactive compound in each fraction. According to Loomis (1978), that the differences in toxic activity by any compound, since each compound will activate specifically to its target. The different formation on the resistance zone diamater of the seven fraction as the result of OCC was due to different defence mechanism. Each bacteria has their own defence mechanism to their environmental stresses, such as the presence of antibiotics. Irianto (2006)

stated that some factors will affect *in vitro* antibacterial activity such as pH, bioactive component, drug stability, inoculum size, incubation time and aktivitas metabolisme activity of microorganisms. GC-MS analysis on fraction IV revealed that this fraction had performed a better antibacterial activity until 72 hours incubation time. Furthermore this fraction had exhibiting 6 different GC-MS peaks, meaning that the fraction have 6 bioactive compounds. This analysis has also identified 5 compounds as 2-methil buthanoate; 2-butoxi ethanol; 3,5,5 trimethile sicloheksan-1; phenil propanadioate; 2-metoxi-4-(2-prophenil) or eugenol.

Bioactive compound had been identified in fraction IV with a significant antibacterial activity as 2-metoxi-4-(2-prophenil) or eugenol. Nakamura *et.al*. (1999) stated that eugenol in *Ocimum gratissimum L*. which has an antibacterial activity. According to Siswandono and Sukardjo (1995), that eugenol with 82 % content in the clove oil, was widely used as antiseptic in the mouth wash and analgetic treatment for teeth. The presence of metoxi compound function as analgetic and anesthetic. Simasatikul *et.al*. (2008), that eugenol (C_{10} H_{12} O_2), such as 2-metoxi-4-(2-prophenil) as group of alilbenzena compound.

CONCLUSIONSS

Etile acetate exctract of *H. Leucospilota* produce 7 (seven) fraction, whichbacterial activity to tested MDR bacteria *Klebsiella* sp. and *Enterobacter*-10. Average resistance zone response by fraction III with concentration 80 µg/disc, resistance zone diameter for *Klebsiella* sp and *Enterobacter*-10 were 11.87 ± 0.90 mm and 11.49 ± 0.86 mm. GC-MS analysis of fraction IV bad identified 5 bioactive compound 2-mehtil butanoat; 2-butoxi ethanol; 3,5,5 trimetil sicloheksan-1; phenil propanadioat; 2-metoxi-4-(2-prophenil) or eugenol. Eugenol or 2-metoxi-4-(2-prophenil) was the most potential candidate as antibacterial for MDR (*Multi Drug Resistant*) bacteria.

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