International Research Journal of Microbiology (IRJM) (ISSN: 2141-5463) Vol. 7(1) pp. 001-009, February, 2018
Available online http://www.interesjournals.org/IRJM
DOI: http:/dx.doi.org/10.14303/irjm.2017.020

Full Length Research Paper

Screening of secondary metabolite from waterfall and marine bacteria as biocontrol agent

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Abstract

Biocontrol agent often used to reduce sources of disease, such as bacteria, fungi, and mosquito. Emergence of antibiotic-resistant bacteria and fungi are increasing due to excessive used of antimicrobial substances. Chemical substances used to kill mosquito larvae in water environment might also be dangerous for human and environment. Hence, a biocontrol agent is required to be safe and effective. The purpose of this study is to discover bacteria from waterfall and marine water which has a good potential as biocontrol agent. Four waterfall bacteria (S 1.1, S 2.1, S 3.1, and S 3.2) and three marine bacteria (A 1.1, A 1.2, and PA 1.3C) were proved to have antifungal activity towards pathogenic fungal. PA 1.3A was the only marine bacteria which proved to have antimicrobial activity towards *Escherichia coli* and *Streptococcus agalactiae*. Extraction of antimicrobial substance using several solvents resulted in larger clear zone diameter and inhibition of other bacteria, such as *Bacillus licheniformis* and *Staphylococcus aureus*. Extracted antimicrobial substance of PA 1.3A was also proved to be non toxic towards *Aedes aegypti* larvae through larvicidal assay and mammals through brine shrimp lethality assay (LC_{50} > 1000µg/mL).

Keywords: Marine bacteria, Waterfall, Antimicrobial, Biocontrol agent, Brine shrimp.

INTRODUCTION

Biocontrol agent utilize natural resources to reduce source of disease. Therefore, it considered to be less harmful towards human and environment (Cook, 1988). Chemical agent might proved to be more effective than biocontrol agent, but for environment biocontrol agent was proved to be more safe, stable, and long-lasting (Sharma *et al.,* 2013). Surfactin produced by *Bacillus subtilis* is one of example of biocontrol agent which is derived from bacteria (Plaza *et al.,* 2013).

Antifungal agent is used to inhibit or kill pathogenic fungi. Usually antifungal agent works by inhibiting fungal cell wall component production, such as chitin and glucan, inhibiting protein, RNA, and DNA synthesis, also interfering with pyrimidine metabolism. Example of antifungal agents are 5-fluorocytosine which inhibit *Candida* and *Cryptococcus neoformans* through protein synthesis disruption and echinocandines which inhibit *Aspergillus* through inhibition of β-glucan synthesis. Nowadays, antifungal-resistance fungi is emerging through various mechanisms such as, mutation and reduce uptake of antifungal agent (Ghannoum and Rice, 1999).

Antibacterial agent is used to inhiit or kill pathogenic bacteria, usually it works through disruption of bacterial cell wall formation or inhibiting DNA synthesis. Antibacterial agent might derived synthetically such as fluoroquinolone and nalidixic acid (Soni, 2012) or from natural resources such as *Bacillus subtilis* bacteria which produced bacteriocin (Joseph *et al.,* 2013) and fungi *Penicillium rubens* which produced penicillin (Houbraken et al., 2011).

Mosquitoes are known as disease-transmitter, such as *Aedes aegypti* and *Anopheles gambiae* that transmitted malaria disease (Derua *et al.,* 2012). Generally, chemical substance like pyrethroids and carbamate are used to kill mosquito, but those substances are harmful towards other insects.

Moreover, due to modification of acetylcholinesterase,

mosquitoes can be resistance towards those chemical substances (Darriet *et al.,* 2005).

Brine shrimp (*Artemia* sp.) is a mammalian model used in toxicity preliminary assessment. It is commonly used due to simplicity, inexpensive, rapid, and only small amount of substance is required (Ajoy and Padma, 2013; Olowa and Nuneza, 2013). Drawbacks of brine shrimp usage is that result might effected by solvents used (Wu, 2014).

Previous study done by Jeganatan *et al.* (2013) proved that bacteria isolated from marine environment (*Alteromonas, Bacillus, Marinobacter*) were able to inhibit pathogenic bacteria such as *Escherichia coli* and *Staphylococcus aureus*. Gohel *et al.* (2004) also proved that some marine bacteria from Bhavnagar (India) were able to produce chitinase which inhibit growth of pathogenic fungi such as *Fusarium*. Marine bacteria also can be used as biocontrol agent towards mosquitoes, as proved by Mani *et al.* (2015). Hence, it can be concluded that bacteria derived from aquatic environment might be useful as biocontrol agent.

MATERIALS AND METHODS

Bacteria isolation from waterfall and marine samples.

Water sample were obtained from overhang parts of waterfall and 300 m range from seashore (Table 1). Each sample were diluted and spreaded into Nutrient Agar (NA) (Oxoid) for waterfall sample (Odeyemi *et al., 2013*) and Marine Agar (MA) (Difco) for marine sample (Jeganathan *et al.,* 2013). All media were incubated at 28^{o}C overnight and different colonies were picked.

Antifungal Assay

Antifungal assay were done with two different methods, agar plug method and disk diffusion method. Pathogenic fungi used are *Aspergillus* sp., *Fusarium* sp., and *Penicillium* sp. Agar plug method was done based on Abla *et al.* (2015). Pathogenic fungi were grown in Potato Dextrose Agar (PDA) (Oxoid) supplemented with chloramphenicol (Kimia Farma) at 30^{o}C for 7 days. Meanwhile, bacterial isolates were grown in PDA medium (without chloramphenicol) at 28^{o}C for 48 hours. Each 4mm plug of pathogenic fungi was put in the middle of media with grown bacterial isolates. All media were incubated at 30^{o}C for 7 days to observed fungal growth inhibition.

Disk diffusion method was done with modification method according to Nweze *et al.* (2010). Pathogenic fungi were grown in Potato Dextrose Broth (PDB) (Pronadisa) medium supplemented with chloramphenicol at 30^{o}C for 7 days. Bacterial isolates were grown in Nutrient Broth (NB) (Oxoid) medium (for waterfall bacteria), Sea Water Complete (SWC) (bacteriological peptone (Oxoid) 5 g/L; yeast extract (Oxoid) 1 g/L; glycerol 85% (Merck) 3 mL/L; 3% NaCl (Oxoid) solution v/v) medium (marine bacteria), and Brain Heart Infusion Broth (BHIB) (Oxoid) medium (waterfall and marine bacteria) at 28^{o}C for 48 hours. Bacteria supernatant were obtaine by centrifugating culture isolate twice at 13,684 ×g (Thermo Scientific) for 10 minutes. Then, sterile cotton swab dipped in each pathogenic fungi was streaked to MHA medium. For positive control, 10µL of Ketoconazole (1mg/mL) (Kimia Farma) and Griseofluvin (1mg/mL) (Darya-Varia) were inserted into blank discs. For negative control, control well was filled with sterile NB, SWC, and BHIB medium. All plates were incubated at 30^{o}C for 7 days. Each treatment was repeated twice.

Antibacterial Assay

Antibacterial assay was done using well diffusion method which modified from method based on Pundir *et al.* (2013). Test bacteria used were grown in Luria Broth (LB) (NaCl (Oxoid) 5g/L, yeast extract (Oxoid) 5g/L, tryptone (Oxoid) 10g/L) at 37^{o}C (*Acinetobacter baumannii* ATCC 19606*, Escherichia coli* ATCC 4157, *Pseudomonas aeruginosa* KCTC 1637, *Salmonella enterica* ATCC 51741*, Staphylococcus aureus* ATCC 25923, *Streptococcus agalactiae* BAA-611), meanwhile *Bacillus licheniformis* ATCC 12759 and *Burkholderia cepacia* ATCC 25416 were grown in LB medium at 30℃ until the absorbance value reach 0.132 at λ=600 nm (McFarland 0.5). Each isolate was grown in NB medium (waterfall isolates) and SWC medium (marine isolates) at 28^{o}C for 48 hours. Isolate suspension was centrifugated twice at 13,684 xg (Thermo Scientific) for 10 minutes to obtained crude extract.

Each test bacteria suspension was spotted and streaked on Mueller-Hinton Agar (MHA) (Oxoid). 4mm well was made using cork borer. Antibiotic discs of nalidixic acid (30 µg/mL) (BBL) and trimethoprim-sulfamethoxazole (25 µg/mL) (Oxoid) were used as positive control, meanwhile sterile NB and SWC medium were used as negative control. 20 µL of crude extract was loaded into each well. Plates were incubated at optimum temperature of each test bacteria. Each treatment was repeated twice.

Extraction of Antimicrobial Substances

Solvents used for extraction of antimicrobial substances were chloroform (Merck) and dichloromethane (Merck). Positive bacteria isolate was grown in SWC medium for 48 hours and measured until the absorbance value reach 0.132 at 600nm. 25 mL of isolate was moved to 1L sterile SWC medium and incubated for 48 hours. Isolate suspension was centrifugated twice at 10,016 ×g

Table 1 Waterfall and Marine Samples Sources

Sample	Sources	Location	Isolates Code	Number of Isolates
Waterfall	Mata Air Darmaloka (1st stage)	Kuningan, West Java	S	10
	Mata Air Darmaloka (7th stage)	Kuningan, West Java	SN	8
	Curug Bibijilan	Sukabumi, West Java	CB	6
	Curug Cikahuripan,	Bandung, West Java	AN	2
	Mata Air Sawer Rahmat	Kuningan, Cirebon, Jawa Barat	SC	12
Marine	Pramuka Island	Kepulauan Seribu, DKI Jakarta	N	10
	Perawan Beach	Kepulauan Seribu, DKI Jakarta	V	14
	Tanjung Pasir Beach	Kepulauan Seribu, DKI Jakarta	A	2
	Bunaken Sea	Manado, North Sulawesi	B	4
	Padang-Padang Beach	Kuta, Bali	SS	2
	Matras Beach	Bangka Island, Bangka Belitung	WM	4
	Ketawai Beach	Bangka Island, Bangka Belitung	WK	5
	Rambak Beach	Bangka Island, Bangka Belitung	WR	2
	Putri Beach	Kepulauan Seribu, DKI Jakarta	LP	4
	Air Island	Kepulauan Seribu, DKI Jakarta	PA	6

(Thermo Scientific) for 10 minutes. Separation of organic phase was conducted using separating funnel with 1:1 (v/v) ratio of isolate supernatant and solvent. Rotary evaporator was used to obtained crude extract from organic phase at 50°C. Crude extract was air dried overnight (Shetty *et al.,* 2014) and diluted into 750 µL phosphate buffered saline (PBS) (NaCl (Oxoid) 8g/L, KCl (Merck) 0.2 g/L, Na_2HPO_4 (Merck) 1.44 g/L, KH_2PO_4 (Merck) 0.24 g/L). Antibacterial activity of crude extract towards pathogenic bacteria was tested again using well diffusion method (Pundir *et al.,* 2013).

Larvicidal Assay towards *Aedes aegypti* larvae.

Larvicidal assay method was modified from Nabar and Lokegaonkar (2015). *Aedes aegypti* eggs were obtained from Faculty of Veterinary, Bogor Agricultural University. Eggs were submerged into 500 mL water for 7 days to allow the eggs hatching into larvae. Tests on each sample extracts were performed in triplicates. Each sample extract was diluted into three concentrations, 500, 1000, and 1500µg/mL with addition of PBS. 0.5 mL of each sample dilution was added in a tube which contained 10 *A. aegypti* larvae in 4.5 mL water. Addition of 0.5 mL water without any sample extract, 0.5 mL solvent (chloroform and dichloromethane), and 0.5 mL supernatant of isolate was also tested as control. Each test was performed triplicates. Larvae mortality was observed after 24 and 48 hours. Mortality percentage was then counted to obtained LC_{50} values.

Brine Shrimp Lethality Assay (BSLA)

BSLA method was modified from Olowa and Nuneza (2013). Cysts (brine shrimp eggs) was hatched in artificial seawater (38 grams of sea salt diluted in 1 liter of water) with continuously illuminated condition using fluorescent lamp and continuous aeration with air pump for 48 hours at room temperature. 10 nauplii (brine shrimp larvae) were added into each test tube with 4.5 mL of artificial seawater. 0.5 mL of sample extract was added. 0.5 mL of artificial seawater, 0.5 mL Phosphate Buffered Saline (PBS), 0.5 mL solvent (chloroform and dichloromethane), and 0.5 mL supernatant were added as control. Each test was performed triplicates. All test tubes was incubated under constant illumination and observed after 24 and 48 hours. Number of surviving nauplii were counted to find out mortality percentage (%M) which then used to count LC_{50} values with statistical analysis. Significance differences was analyzes using ANOVA and TUKEY test. LC_{50} values greater than 1000µg/mL is considered non-toxic, meanwhile LC_{50} values less than 1000 µg/mL is considered as toxic (Meyer *et al.,* 1982).

RESULTS

There are total of 91 isolates derived from waterfall and marine samples. Screening of antifungal assay was done in agar plug method and disk diffusion method. Agar plug method showed only A 1.2 isolate from Tanjung Pasir beach inhibit growth of *Penicillium* sp. (Figure 1). Disk diffusion method showed more diverse result, such as S 1.1, S 2.1, S 3.1, and S 3.2 isolates from 1st stage of Darmaloka spring water which has various inhibition capability towards all three pathogenic fungal (Table 2). Isolate PA 1.3C from Air Island and A 1.1 from Tanjung Pasir beach also showed inhibition towards *Trichoderma* sp. (Table 3). Isolate S 1.1 and S 3.1 showed larger

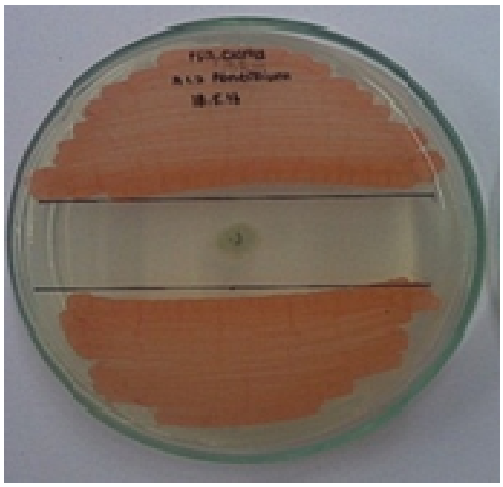

Figure 1. Antifungal activity (agar plug method) of isolate A 1.2 towards *Penicillium* sp.

Table 2 Antifungal activity results of waterfall isolates (disk diffusion method)

Pathogenic Fungal	Growth media	Bacterial Isolates	Diameter of Inhibition Zone (mm)	
			MHA	PDA
Aspergillus sp.	NB	S 1.1	3.00	-
		S 2.1	-	-
		S 3.1	4.00	-
		S 3.2	3.50	-
	BHIB	S 1.1	2.25	-
		S 2.1	-	-
		S 3.1	3.75	-
		S 3.2	1.75	-
Trichoderma sp.	NB	S 1.1	10.50	8.50
		S 2.1	2.50	-
		S 3.1	7.00	8.50
		S 3.2	3.50	-
	BHIB	S 1.1	7.00	11.50
		S 2.1	-	-
		S 3.1	6.00	16.00
		S 3.2	2.25	-
Penicillium sp.	NB	S 1.1	6.50	6.00
		S 2.1	1.00	-
		S 3.1	5.50	6.50
		S 3.2		
	BHIB	S 1.1	4.50	7.00
		S 2.1	-	-
		S 3.1	4.00	7.00
		S 3.2	-	-

inhibition zone in PDA medium compared to MHA medium.

Out of 91 isolates, only one isolate derived from Air Island (PA 1.3A) showed antibacterial activity through inhibition of *Escherichia coli* (Figure 3) and *Streptococcus agalactiae* with inhibition 8.5 mm and 6.5 mm inhibition

Table 3. Antifungal activity results of marine isolates (disk diffusion method)

Pathogenic Fungal	Growth media	Bacterial Isolates	Diameter of Inhibition Zone (mm)	
			MHA	PDA
Aspergillus sp.	SWC	PA 1.3A	-	-
		PA 1.3C	-	-
		A 1.1	-	-
		A 1.2	-	-
	BHIB	PA 1.3A	-	-
		PA 1.3C	-	-
		A 1.1	-	-
		A 1.2	-	-
Trichoderma sp.	SWC	PA 1.3A	-	-
		PA 1.3C	2.00	-
		A 1.1	1.75	-
		A 1.2	-	-
	BHIB	PA 1.3A	-	-
		PA 1.3C	2.50	-
		A 1.1	2.50	-
		A 1.2	-	-
Penicillium sp.	NB	PA 1.3A	-	-
		PA 1.3C	-	-
		A 1.1	-	-
		A 1.2	-	-
	BHIB	PA 1.3A	-	-
		PA 1.3C	-	-
		A 1.1	-	-
		A 1.2	-	-

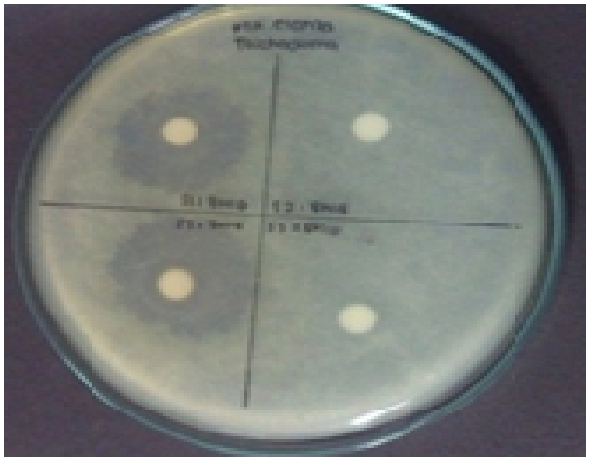

Figure 2. Antifungal activity (disk diffusion method) of S 1.1 and S 3.1 isolates towards *Trichoderma* sp. in PDA medium.

zone respectively. Extraction of antibacterial substances from PA 1.3A isolate using organic solvents showed that more bacteria were inhibited and inhibition zone diameter towards *S. agalactiae* was increased (Table 4).

Larvicidal assay results of PA 1.3A extract showed that there were no significance differences between each

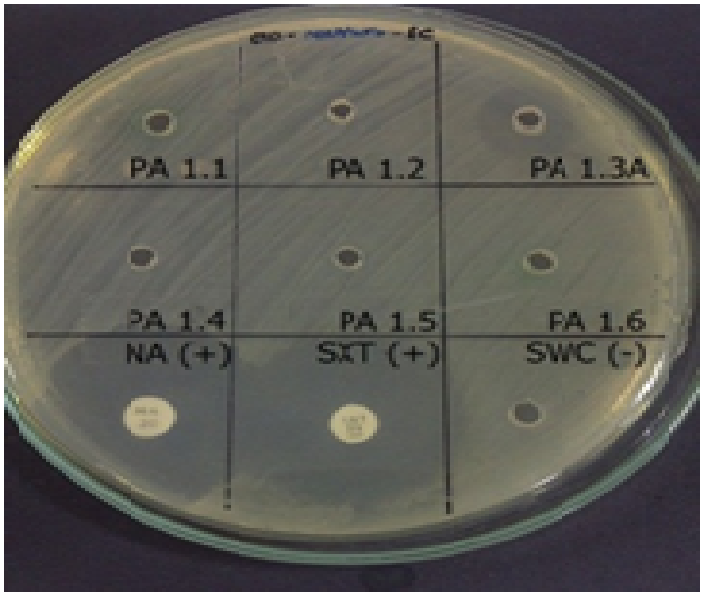

Figure 3. Antibacterial activity shown by PA 1.3A isolate towards *Escherichia coli.* PA = Isolate from Air Island; NA = Nalidixic acid; SXT = Trimethoprim-sulfomethoxazole; SWC = Sea Water Complete broth

Table 4 Antibacterial activity results of PA 1.3A extract

Pathogenic Bacteria	Diameter of Inhibition Zone (mm)	
	Chloroform	Dichloromethane
S. agalactiae	7.50	8.5
S. aureus	2.25	5.0
B. licheniformis	0.50	6.0

Table 5. Larvicidal assay of PA 1.3A extract

Bacterial Isolate	Period (Hour)	Solvent	% Larva Mortality			LC_{50} Value (mg/L)
			Extract Concentration (µg/mL)			
			500	1000	1500	
PA 1.3A	24	Chloroform	13.3	13.3	20.0	1337.23
		Dichloromethane	6.7	20.0	30.0	0.09
	48	Chloroform	16.7	23.3	33.3	0.06
		Dichloromethane	16.7	23.3	33.3	0.06

concentrations and all of them were not toxic towards *Aedes aegypti* larvae (Table 5). PA 1.3A extract also proven to be non-toxic towards brine shrimp, which was shown by LC_{50} values that was higher than 1000µg/ml (Table 6). There were no significance differences between all three concetrations towards nauplii mortality.

Table 6. Brine shrimp lethality assay of PA 1.3A extract

Bacterial Isolate	Solvent	% Nauplii Mortality Extract Concentration (µg/mL)			LC_{50} Value (µg /mL)
		500	1000	1500	
PA 1.3A	Chloroform	13.3	23.3	30.0	40938.41
	Dichloromethane	3.3	6.7	43.3	2444.76

DISCUSSION

Antifungal assay was done in different methods in order to compared both methods. Agar plug assay results showed only one isolate which had antifungal activity towards *Penicillium* sp., meanwhile disk diffusion method showed diverse antifungal activity towards all three pathogenic fungal (*Aspergillus* sp., *Trichoderma* sp., *Penicillium* sp.). Agar plug method was often used for screening of antifungal activity because it is a simple, low-cost method, and enormous number of microorganisms can be tested (Balouiri *et al.,* 2016). Disk diffusion method was commonly used to screen extracellular antifungal activity. Although, it also can be used to screened intracellular activity with usage of bacterial pellet (including cell). Bacterial pellet can be concentrated with centrifugating bacterial culture or reducing pressure towards culture with sonicator (Fawzy *et al.,* 2011; Pandey and Malviya, 2014). In this study, it can be concluded that disk diffusion method was a better method to screened extracellular antifungal activity and agar plug method was better to screened intracellular antifungal activity.

Two kinds of media, Mueller-Hinton Agar (MHA) and Potato Dextrose Agar (PDA) were used in disk diffusion method. Both media were used to compared result between antifungal activity. PDA media is commonly used for disk diffusion method, but Nweze *et al.* (2010) proved that MHA media can also be used for disk diffusion method because it could enhance clear zone visual. MHA media was generally acceptable for antimicrobial test, because it provide stable and reproducible result (Murray and Zeitinger, 1983). In this study, more isolates shows variation of inhibition zone in MHA media, but some isolates (S 1.1 and S 3.1) showed larger clear zone in PDA media.

Bacterial isolates were grown in three kinds of media, Nutrient Broth (NB) for waterfall isolates, Sea-Water Complete (SWC) for marine isolates, and Brain Heart Infusion Broth (BHIB) for both waterfall and marine isolates. Result showed that S 1.1 and S 1.3 isolates (waterfall sources) produced more antifungal substances (which can be seen with larger clear zone) in BHIB media than NB media. A 1.1 and C isolates (marine sources) also produced more antifungal substances in BHIB media than SWC media. BHIB media is considered to be a nutrient-rich media compared to NB and SWC media (Jaradat and Bhunia, 2002). Nutrient-rich condition in BHIB might increase growth rate of bacteria which made bacteria moved to stationary phase faster and produce more secondary metabolites as antifungal substances. Similar result was also shown by Marinho *et al.* (2009) where BHIB supported growth of bacteriocin producing *Pseudomonas putida.* Another study by Elfalah *et al.* (2013) also showed that antimicrobial activity of actinomycetes derived from marine environment, showed highest inhibition when actinomycetes was cultured in BHIB medium.

Antibacterial activity was only shown by PA 1.3A isolate towards *Escherichia coli* and *Streptococcus agalactiae*. PA 1.3A extract only has antibacterial capability towards all gram positive bacteria used in this study, which were *S. agalactiae, S. aureus,* and *B. licheniformis*. Hence, PA1.3A extract has no activity towards *E. coli* which is a gram negative bacteria. This could happened because gram positive bacteria are commonly more sensitive towards antibacterial substances compared to gram negative (Hooper, 2001). In this study, chlorofom and dichloromethane were used because previous study done by Veronica *et al.* (2014) showed that antibacterial compound can be extracted using chloroform and dichloromethane. Extraction result was depended on polarity of antibacterial compound (Sunder *et al.,* 2011). Therefore, to enhance antibacterial activity of PA 1.3 A extract different solvents can be used to explore inhibition activity towards different grams of bacteria.

Bashir et al. (2015) proved that chloroform can be used to extract antibacterial substances from ginger. Chloroform usually attract flavonoid and steroid from ginger. Chloroform could attract lipopeptides from *Bacillus* which inhibit growth of *Saccharomyces cerevisiae* and *E. coli* (Salam *et al., 2015*). Bazzaz *et al.* (2013) proved that dichloromethane was also used to extract antibacterial substance from *Scutellaria lindbergii* Rech.f. and dichloromethane attract more flavonoid but less phenolic substances. Hence, different types of solvents can be used to extract and showed diverse results.

Differences of bacterial cell wal composition in gram positive and gram negative bacteria also played an important role in susceptibilities of gram positive bacteria

towards antibacterial substances. Gram positive bacteria cell wall consist of thick layes of peptidoglycan and cell membrane, but without an outer membrane. Meanwhile, gram negative bacteria cell wall has an outer membrance which consists mostly lipopolysaccharide (LPS) and thin layer of peptidoglycan. This outer membrane played a crucial role as a barrier from harmful substance and stabilizing inner membrane of gram negative bacteira. Gram negative bacteira also has a transenvelope machine which act as efflux pump that expels harmful molecules such as antibiotics (Silhavy *et al.,* 2010).

All three extract concentration (500, 1000, 1500 µg/mL) proved to be non-toxic towards *A. aegypti* larvae. LC_{50} value of chloroform extract was 1337.23 mg/L and 0.06 mg/L for 24 and 48 hours respectively, meanwhile for dichloromethane extract LC_{50} value in 24 hour was 0.09 mg/L and in 48 hours was 0.06 mg/L. Therefore, it can be concluded that PA 1.3A extract concentration must be above 1500µg/mL for larvicidal activity. Further study is needed to find out exact concentration of PA 1.3A extracts which has larvicidal effect (toxic towards *A. aegypti* larvae).

Mosquito will reach larval stage during one week after eggs were submerged in water. Larval stage was used in this study because at this stage larvae cannot move out from breeding sites and harmless (not acquisited with disease) (Hardin and Jackson, 2009). Eggs state was not used because eggs are usually immobile (concentrated on one area) and it will be more difficult for substances to reach the eggs. Hence, larval stage give more advantage because mosquito was spreaded in water and reachable by substances (Elimam *et al.,* 2013).

Extract concentration can be increased by using different solvent in order to improved larvicidal ability of PA 1.3A extract. Usage of more polar substance for extraction is suggested to produce more variety of PA 1.3A extract activities (Arasu *et al.,* 2014). Example of bacterial used as biocontrol agent was *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) which was effective towards mosquito larvae (Priest, 1992).

According to Meyer *et al.* (1982), LC_{50} value higher than 1000µg/mL would be considered as non-toxic substances. In this study, LC_{50} value derived from brine shrimp lethality assay of 500-1500µg/mL concentration proved that PA 1.3A extract is not-toxic for human use. If further study find out about larvicidal conentration of PA 1.3A extract, then PA 1.3A extract concentration toxicity towards brine shrimp also need to be tested.

Example of mosquito biocontrol is cyanobacteria extract which were tested towards A. aegypti larvae and brine shrimp. LC50 value of cyanobacteria extract towards mosquito larvae is 20.8 8µg/mL and has a dose-dependent characteristic (higher doses, higher mortality rate).

Purified substances was also not toxic towards brine shrimp. Hence, cyanobacteria has a good potential to produce non-toxic biocontrol agent (Berry, 2014).

CONCLUSION

Out of 91 isolates, four isolates from 1st stage of Darmaloka water springs (S 1.1, S 2.1, S 3.1, S 3.2), 2 isolates from Tanjung Pasir beach (A1.1 and A 1.2), 1 isolate Air Island (PA 1.3C) were able to inhibit growth of various pathogenic fungal. One isolate (PA 1.3A) from Air Island has antibacterial ability towards *Escherichia coli* and *Streptococcus agalactiae.* PA 1.3A extract derived from extraction with chlorofom and dichloromethane was more effective towards gram positive bacteria (*Bacillus licheniformis*, *Streptococcus agalactiae*, *Staphylococcus aureus*).

PA 1.3A extract in three concentrations was not toxic both towards *Aedes aegypti* larvae and brine shrimp. Therefore, further test is still needed to find out exact concentration of PA 1.3A extract which is toxic towards *A. aegypti* larvae and safeness of extract concentration with toxicity test towards brine shrimp.

ACKNOWLEDGEMENT

This research was funded by the Atma Jaya Catholic University of Indonesia Research Centre.

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