

The Identification of Chemical Compound and Antibacterial Activity Test of Kopasanda (*Chromolaena Odorata* L.) Leaf Extract Against *Vibriosis*-Causing *Vibrio harveyi* (MR 275 Rif) on Tiger Shrimp

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Abstract

Kopasanda (*Chromolaena odorata* L.) R.M King and H. Rob were classified as blossoming bush species in the Asteraceae family. An empirical study showed that kopasanda (*C. odorata* L.) leaf had been used by the embankment farmers at South Sulawesi Province to eradicate *Vibriosis* which caused death for tiger prawn (*P.monodon* Fabr.). The objective of research was to understand the bioactive compound that was contained within kopasanda (*C. odorata* L.) leaf extract and its antibacterial activity against *V. harveyi*. Result of

identification showed that the presence of phenolic, flavonoid, alkaloid, and steroid. Result of the extraction of 500 g kopasanda (*C. odorata* L.) leaf powders with methanol solvent was producing 81.010 g methanol extracts. Its antibacterial test against *V. harveyi* indicated that there was an ability to prevent the growth of *V. harveyi*. The partition of 50 g methanol extract with ethyl acetate was resulting in 29,605 g ethyl acetate extract. The residue was partitioned with n-hexane which produced 9,575 g n-hexane. These three extracts were subjected to the test of antibacterial activity against *V. harveyi*. Result of antibacterial activity test showed that the methanol extract was producing the highest preventive zone, which was 19 mm. It was followed by 9 mm from the ethyl acetate extract, while n-hexane extract did not show a preventive zone at all. The test indicated minimum inhibitory levels (MIC), supporting the conclusion that the kopasanda (*C.odorata* L.) leaf extract inhibited the growth of *V. harveyi* active with a minimum inhibitory concentration of 0,625 mg / mL.

Keywords: Kopasanda (*Chromolaena odorata* L.) Leaf, Chemical compound, Antibacterial, *Vibrio harveyi*, Tiger Shrimp (*Penaeus monodon* Fabr.)

1. Background

A main problem of the tiger shrimp cultivation, was a mass mortality due to disease attack. One such disease was considered as a serious problem, which caused by pathogenic bacteria infection, especially from vibrio genus, called *Vibrio harveyi* (Mariyono *et al.*, 2002). These bacteria infect almost all cultivated marine organisms such as crustacean, mollusca and fish (Isnansetyo *et al.*, 2009). Crustacean, including shrimp, crab, lobster and *Artemia*, very susceptible to this opportunistic pathogenic bacteria (Sunaryanto & Maryam, 1986; Lavilla-Pitogo *et al.*, 1992; Jivaranichpaisal *et al.*, 1994; Karuna sagar *et al.*, 1994; Liu *et al.*, 1996; Robertson *et al.*, 1998; Diggles *et al.*, 2000; Soto-Rodriguez *et al.*, 2003; and Bourne *et al.*, 2007). Result of *in vivo* study indicated that *V. harveyi* was pathogenic to the post-larva stage (PL-14) of prawn in the density of 10^7 CFU/mL (Khodriah *et al.*, 2011).

A general way to deal with the vibriosis disease was using chemical and antibiotic substances (Brown, 1989). These substances, bacteria however have become resistant to these chemical which are, also harmful to the environment, and accumulate in shrimp which then influenced the quality of shrimp and become hazardous to the consumer (Defoirdt *et al.*, 2007). As such, countries in European Union in recent years have implemented a *zero tolerance* to fishery product, including prawn (Fattah, 2008) treated with these chemicals harming Indonesian exports and calling for less harmful methods of treating vibriosis disease.

One proposed alternative is the use of kopasanda (*Chromolaena odorata* L.) leaf extract (Suryati and Gunarto 2001) due to high levels of bioactive compound with natural antibacterial activity. Other uses for leaf extracts include healing injured skin and blood retainer (Anonymous, 2012). Other parts of the plant also have medicinal uses. For example the root is also useful as anti-pyretic and analgesic agents. The flower of the plant might be usable as insect poison (Anonymous, 2012). Research by embacment farmer in South Sulawesi suggest kopasanda plant control shrimp disease. (Suryati and Gunarto, 2001).

Here we report firstly the chemical properties and the antibacterial activity of kopasanda (*C.odorata* L) leaf extract against vibriosis-causing *V. harveyi* at post-larva stage of tiger shrimp examining the potential of kopasanda plant as the natural anti-bacterial substance in the elimination of vibriosis on tiger shrimp

2. Material and Method of Research

2.1 Sampling and Identification of Kopasanda (*C. odorata* L.) Plant

Kopasanda plant (*C. odorata* L.) were sampled from The Experiment Embankment Installation Area of Research Institute for Coastal Aquaculture, Maranak, Maros District, South Sulawesi, Indonesia in June 2012 (Figure 1).



Figure 1. Sampling Location of Kopasanda (*C. odorata* L.)

Kopasanda (*C.odorata* L.) identification were confirmed at “Herbarium Bogoriense” Laboratory, Botanical Division of Research Center for Biology- Indonesian Institute of Sciences-Bogor Voucher No. 536/IPH.1.02/If.8/IV/2012. The specimen was stored there.

2.2 The Preparation, Extraction and Partition of Kopasanda (*C. odorata* L.) Leaf

Sample preparation was undertaken at the Biofarmaka Laboratory, Faculty of Pharmacy, University of Hasanuddin. Here, kopasanda leaf samples were washed through the flowing water and dried by aerating. 500 grams of kopasanda leaf powder was extracted by soaking the whole simplicia in 96% methanol solvent (MeOH) and incubating for 3 x 24 hours. Then, methanol extract and its dregs were separated. Liquid methanol extract from kopasanda leaf was vaporized to lift out methanol using rotary evaporator at 40⁰C. The viscous methanol extract was then obtained and ready for antibacterial activity screening test. The dregs were dried and weighted to figure out its final weight. Moreover, 50 grams viscous methanol extract was partitioned into Solid-Liquid partition using n-hexane solvent to obtain n-hexane extract and residue. The residue was then partitioned again into Solid-Liquid partition using ethyl acetate solvent to produce ethyl acetate extract. Subsequently, these three samples of viscous extract (methanol, n-hexane, and ethyl acetate extracts) were tested for its antibacterial activity against *V. harveyi* using disc diffusion method (Bauer *et al.*, 1966)).

2.3 Phytochemical Test of Kopasanda (*C.odorata* L) Leaf Extract

Phytochemical test was conducted to understand the bioactive compounds within kopasanda (*C. odorata* L.) leaf, which included phenolic, tannine, steroid, saponin, flavanoid, and alkaloid. The procedure to analyze each bioactive compound was as followed:

1) Phenolic Compound Test, 0.1 g of extract sample was dissolved into 1 ml methanol. Few drops of FeCl₃ 1% were given. The presence of phenol was understood by the color of purple, green and black (Wibowo *et al.*, 2008; Chulet *et al.*, 2010).

2) Tannin Compound Test, FeCl₃ Test. 2 grams of sample were extracted with 5 ml hot water. The extract was screened by cotton and moved into other tube. Two to three drops of

FeCl_3 1% were added. The sample that was positively containing tannin would change its color into blackish green (IAEA, 2000; Chullet *et al.*, 2010)

3) Flavonoid Compound Test

- Shinoda Test. The sample was dropped by alcohol and added by Mg powder, and dropped again with viscous HCl. Flavonoid was shown through colors such as orange (flavon), red (flavonol), and purple (xanthon) (Wibowo *et al.*, 2008; Arulpriya *et al.*, 2010).

- NaOH Test: 2 grams of sample were extracted with 5 ml methanol, screening the extract with cotton, moving it to other tube, adding 2 drops NaOH 10 %, and shaking it strongly. The sample would positively contain flavonoid if there was very salient color.

- H_2SO_4 Test: 2 ml liquid methanol extract and viscous sulfate acid. The presence of flavonoid was shown by the colors of dark yellow, bluish red (chalcone, aurone), and red-orange (flavonon) (Wibowo *et al.*, 2008; Arulpriya *et al.*, 2010).

4) Saponin Compound Test

2 grams of each sample was extracted with 5 ml hot water, screening the extract with cotton moving it to other tube, shaking it strongly and incubated for 2 minutes before 2 drops 2 N HCl were added. Strong shaking was given again to see whether the foam emerged after incubation for 10 minutes. The sample would contain saponin if the foam was highly intensive and consistent for 10 minutes.

5) Alkaloid Compound Test

- Mayer Test. 2 ml of liquid methanol extract was added to two drops of Mayer reactant (reagent). Alkaloid was present by white precipitation. (Preparation of Mayer Reagent was involving two solutions. First was produced by dissolving 1.36 g HgCl_2 into 60 ml aquades. Other solution was made by dissolving 5 g KI into 10 ml aquades. Both solutions were mixed and diluted to obtain 100 ml volume) (Wibowo *et al.*, 2008; Arulpriya *et al.*, 2010).

- Dragendorff Test. 2 ml of liquid methanol extract was mixed with ten drops of chloride acid or viscous sulfate acid. Two drops of Dragendorff reactant (reagent) was added, and the presence of alkaloid was shown by orange precipitation. (Preparation of Dragendorff Reagent also involved two solutions. One solution was produced by dissolving 8 g KI with 20 ml aquades. In other batch, 0.85 g bismuth sub-nitrate ($\text{BiNO}_3(\text{OH})_2\text{BiO}(\text{OH})$) was dissolved into 40 ml glacial acetate acid. Both solutions were mixed together and diluted to achieve 100 ml volume) (O'Brien *et al.*, 1982; Wibowo *et al.*, 2008).

- Wagner Test. 2 ml of liquid methanol extract was mixed with two drops Wagner reactant (reagent). Brown precipitation indicated the alkaloid compound. (Preparation of Wagner Reagent was by dissolving 1.72 g I_2 dan 2 g KI with 5 ml aquades, and diluted to obtain 100 ml volume) (Wibowo *et al.*, 2008; Arulpriya *et al.*, 2010).

6) Terpenoid and Steroid Compounds Test

Lieberman–Burchard Test. 2 ml of leaf sample was mixed with two drops

Lieberman-Buechard Reactant (reagent). Terpenoid was shown by the presence of blue, red and purple colors. (Preparation of Lieberman-Buechard Reagent was involving viscous sulfate acid and anhydrate acetate acid. Both acids were stored in different batch/bottle) (Wibowo *et al.*, 2008; Jagessar & Cox, 2010).

Salkowski Test. 2 ml methanol extract was mixed with 1 ml chloroform and with 1 ml viscous sulfate acid, thus producing two layers. Red or orange layer showed the presence of terpenoid and steroid compounds (Egwaikhide & Gimba, 2007; Wibowo *et al.*, 2008).

2.4 Preparation of *Vibrio harveyi*

Antibacterial activity was tested with pure culture of *V. harveyi* obtained from the isolation of Vibriosis-infected shrimp at Environmental Health Laboratory of Research Institute for Coastal Aquaculture (RICA) Maros District, South Sulawesi, Indonesia. The bacterial culture was prepared using the bacterial rejuvenation procedure (Rosmiati *et al.*, 2011). This procedure involved some rejuvenation stages such as: (1) 5 ml Nutrient Broth (NB) solution were prepared in the reaction tube; (2) *V. harveyi* bacteria pure culture was aseptically taken for 1 oce, and this culture was put into Nutrient Broth (NB) agar; (3) Bacteria-contained Nutrient Broth (NB) solution would be incubated for 24 hours at 37°C; (4) After incubation for 24 hours, the bacteria in the Nutrient Broth (NB) agar were cultured again into solid media using *Thiosulphate Citrate Bile Sucrose Agar* (TCBSA), and incubated again for more than 24-48 hours at 25 °C; and (5) After 24 hours, the bacteria were ready for antibacterial test.

2.5 Antibacterial Potential Test of Kopasanda (*C. odorata* L.) Leaf Extract against *V. harveyi*

The potential of kopasanda leaf extract as the antimicrobial substance was determined by weighting and dissolving 10 mg kopasanda (*C. odorata* L.) leaf extract with 0.2 ml DMSO. The dissolved extract was then given 9.8 ml medium MH (Muller Hinton) to obtain the concentration of 1 mg/mL and subsequently poured into petri dish, and homogenized and solidified. *V. harveyi* suspension was then smeared onto already-solidified MH (Muller Hinton) medium, and this medium was incubated for 1 x 24 hours at 37 °C. The growth of *V. harveyi* was then observed.

2.6 Antibacterial Activity

Antibacterial activity test employed the Kibry-Bauer Method (Bauer *et al.*, 1966) using disc diffusion. Prior to testing, *Vibrio harveyi* inoculums were zig-zag streaked on TCBSA (*Thiosulfat Citrate Bile Sucrose Agar*) and incubated for 24 hours at 28°C. One colony of the bacteria growth was sub-cultured in 50 ml of nutrient broth medium (Sigma, FRG) and incubated for 4 hours using a shaker at 150 rpm and 28°C to produce the density of 10⁷ CFU/mL (Khodriah *et al.*, 2011). Furthermore, 100 µL of bacteria inoculum were spread on Mueller Hinton agar. The filter paper discs (6 mm in diameter) were individually impregnated with 20 µL of the extracts (30 mg/mL), dried in a laminar air flow and then placed onto the agar plates previously inoculated with the tested microorganisms. The plates were incubated at 30 °C for 24 h. The diameters of the inhibition zones were measured in millimetres. All the tests were performed in triplicate. The antibacterial activity was then

interpreted as followed: The diameter of inhibition zone ≥ 15.0 mm was considered as strong; 10.0 to 14.5 mm as moderate and <10 mm as weak (Habsah *et al.*, 2007; Rosmiati *et al.*, 2011).

2.7 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was the smallest antimicrobial concentration which prevented microscopic growth of bacteria. The minimum inhibitory concentration (MIC) of the ekstrak against *V. harveyi* was tested by using serial dilution. Ten (10) sterile reaction tubes were prepared. Kopasanda leaf methanol extract (20 mg/mL) was put into the first tube. The dilution of the extract was conducted from the second tube to the seventh tube, thus producing doses of 10; 5; 2.5; 1.25; 0.625; 0.313; and 0.156 mg/mL. Each tube received 1 oml of *V. harveyi*. The eighth tube received NB + *V. harveyi*, the ninth tube accepted NB without *V. harveyi*, and the tenth tube accommodated NB+ kopasanda leaf extract. All tubes were vortexed and incubated in the incubator for 24 hours at 37°C. After 24 hours, whole tubes were observed to compare the medium viscosity of test samples compared the positive and negative control. Each tube was inoculated into TCBSA (*Thiosulfat Citrate Bile Sucrose Agar*) solid and incubated for 24 hours. The prevention of growth indicated the lack of bacterial colony growth. The lowest dose that was able to prevent the bacterial growth was called Minimum Inhibitory Concentration (MIC).

3. Result

3.1 Result of Extraction and Partition of Kopasanda (*C. odorata* L.) Leaf

Result of Extraction and Partition of Kopasanda (*C.odorata* L.) Leaf was shown in Figure 2.

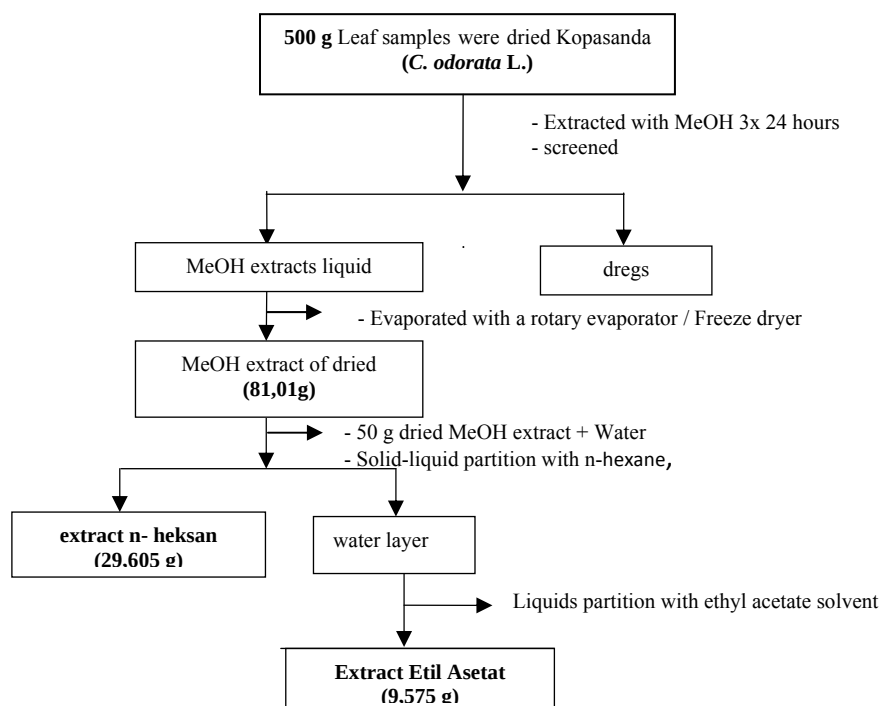


Figure 2. Extraction and partition path Kopasanda (*C.odorata* L) leaf

3.2 Result of Phytochemical Test of Kopasanda (*C. odorata* L.) Leaf Extract

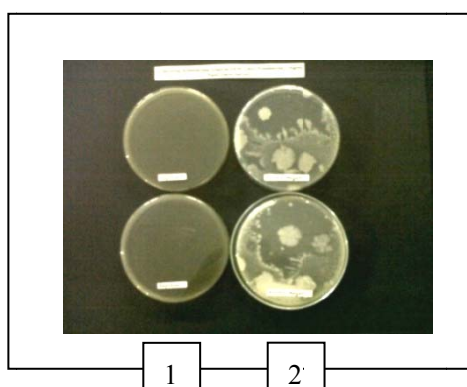
Result of quantitative test of bioactive compound in the kopasanda (*C.odorata* L.) leaf extract was shown in Table 1.

Table 1. Result of phytochemical test of active substance of Kopasanda (*C. odorata* L.) leaf

No	Active Substance	Discoloration	Result
1.	Phenolic	Blackish green	+
2.	Tannin	None	-
3.	Terpenoid and Steroid of Lieberman-Buerchard Reagent	Green	+
4.	Saponin	No foam	-
5.	Flavonoid with reagents		
	HCl	Orange brown, purple	+
	H ₂ SO ₄	Brown	+
	NaOH	Salient green	+
6.	Alkaloid		
	Dragendorff Reagent	Red precipitation	+
	Wagner Reagent	Brownish precipitation	+
	Mayer Reagent	Cream precipitation	+
	Hager Reagent	Yellow precipitation	+

3.3 Result of Antimicrobial Potential Test of Kopasanda (*C. odorata* L.) Leaf Extract against *V. Harveyi*

After testing the antibacterial potential against *V. harveyi*, it was obtained the result that was similar to that in Figure 3 and Table 2.



Particulars 1 : Treatment with kopasanda extract
 2 : Treatment without kopasanda extract

Figure 3. Result of antimicrobial potential test of Kopasanda extract against *V. harveyi*

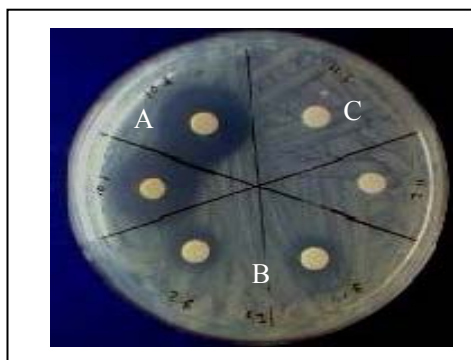
Table 2. Result of *in-vitro* potential test of active compound extract of Kopasanda (*C. odorata* L.) Leaf

No.	Treatments	Replication	Bacterial Growth
1	With kopasanda (<i>C.odorata</i>) extract	1	-
		2	-
2.	Without kopasanda (<i>C.odorata</i>) extract	1	+
		2	+

Particulars: -: No bacterial growth; +: Bacterial growth presented

3.4 Antibacterial Activity of Kopasanda (*C. Odorata* L.) Leaf Extract against *V. Harveyi*

Result of antibacterial activity of kopasanda (*C. odorata* L) leaf extract against *V. harveyi* with different solvents was shown in Figure 3, while the average of inhibitory zone diameter was shown in the histogram form in Figure 4.



Particulars: A. Methanol solvent; B. Ethyl acetate solvent; C. n-hexane solvent

Figure 4. Result of antibacterial activity of Kopasanda (*C. odorata* L.) leaf extract

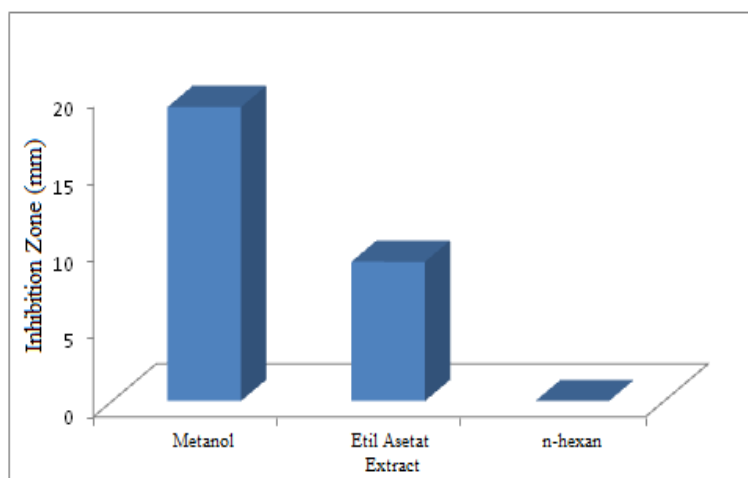


Figure 5. The histogram of inhibition zone (mm) of each extract

3.5 Determination of Minimum Inhibitory Concentration (MIC) of the Active Extracts of *Kopasanda* (*C. odorata* L)

Result of MIC test of methanol extract of *kopasanda* (*C.odorata* L.) leaf was shown in Table 3.

Table 3. Minimum inhibitory concentration (MIC) test of *Kopasanda* (*C. odorata* L) extract against *V. harveyi* growth

No	Extract Doses (mg/mL)	<i>V. harveyi</i> Growth
1	10.000	-
2	5.000	-
3	2.500	-
4	1.250	-
5	0.625	-
6	0.313	+
7	0.156	+
8	NB + <i>V. Harveyi</i>	+
9	NB	-
10	NB + <i>Kopasanda</i> Extract	-

Particulars: + : *V. harveyi* Growth presented; - : No *V. harveyi* Growth.

Table 3 indicated that Minimum Inhibitory Concentration rate of *kopasanda* leaf extract at dose 0,625 mg/mL was actually able to eliminate *V. harveyi*.

4. Discussion

The study showed that *kopasanda* (*C. odorata* L.) leaf is useful as an antibacterial compound against *V. harveyi* to eliminate vibriosis in the prawn cultivation. Result of chemical compound identification indicated that phenolic, flavonoid, alkaloid and steroid were presented, similar to result reported by Akinmoladun, *et al.*, (2007), that *kopasanda* leaf could be developed as natural antibacterial substance.

Phenolic compounds have been shown to disturbed the function of cytoplasm membrane and therefore have been described as antibacterial substances (Volk and Wheeler 1993; Cowan, 1999). At low concentration, phenolic compound can still damage cytoplasm membrane causing the leakage of important metabolite and inactivated bacterial enzymatic system. At high concentration, this compound can damage cytoplasm cell membrane and to precipitate the cell protein. Phenolic compound interact with the component of bacterial cell wall which causes higher permeability of the bacterial cell. This compound also diffuses into the cell slowing and even stopping bacterial growth (Pelczar and Chan, 2005). Moreover, Mori *et al.*, (1987) added that some flavonoid substances such as robinetin, myricetin and epigallocatechin, interfere with the intercalation bond or hydrogen bond at the nucleate acid assembly preventing the activity of DNA and RNA syntheses. Furthermore, steroid compound have been shown to prevent microbial growth by damaging plasma membrane such that the cell cytoplasm was leaked, thus causing cellular death (Putra, 2007).

Antibacterial activity potential test was carried out to ensure whether methanol extract of kopasanda (*C. odorata* L.) leaf had ability to prevent *V. harveyi*. Method used in this test was solid dilution method at concentration of 1 mg/mL. The concentration used was consistent with Hoffman (1991) who said that plant extract could be antimicrobial if its concentration of 1 mg/mL could prevent the growth of *V. harveyi*. Based on the anti bacterial activity potential test in the current study, it was confirmed that kopasanda (*C. odorata* L.) leaf extract prevent the growth of *V. harveyi*. The ability of kopasanda (*C.odorata* L) leaf extract to prevent *V. harveyi* growth was indeed increasing opportunity that *C. odorata* L. plant could be used as antimicrobial in controlling pathogenic microorganism which caused disease at post-larva stage of prawn. Other research has shown that this leaf extract also restricts the growth *Bacillus subtilis*, *Staphylococcus aureus* and *Salmonella typhimurium* (Vital *et al.*, 2009).

Antibacterial power to prevent microorganism growth was different. The determination of antibacterial power based on Davis Stout Method was producing a classification as followed: Inhibitory zone of 2.0 cm or more classified as very strong; 1.0-2.0 cm as strong; 0.5-1.0 cm as moderate; and smaller than 0.5 cm as weak. Habsah *et al.*, (2007) asserted that inhibitory zone diameter ≥ 15 mm was classified as strong, while 10-14.5 mm was classified as moderate and < 10 mm was classified as weak. In this study, Strong inhibitory power was shown by methanol extract with 19 mm inhibition zone, followed by ethyl acetate extract with 9 mm inhibitory zone. There was no inhibitory zone in n-hexane extract. It meant that bioactive compound that was extracted from *C. odorata* L leaf had antibacterial activity, and could be dissolved into polar and semi-polar solvents, but might not dissolve within non-polar solvent. Therefore, bioactive compound could be extracted with methanol solvent. Review of literature showed that solvents in the extraction would destroy cellular membrane and dissolve pigments (Shidduraju & Becker, 2003; Ra *et al.*, 1998; Kahkonen *et al.*, 1999). Non-polar solvent, such as n-heksan, was effective to dissolve alkaloid in the alkali form (Jaiarj *et al.*, 1999; Sosa *et al.*, 2010). Semi-polar solvent, such as ethyl acetate, was able to extract phenol and terpenoid compounds, while polar solvent, like methanol, was able to extract quaternary alkaloid compound, phenolic component, carotenoid and tannin (Harborne, 2006).

Based with the result of minimum inhibitory concentration (MIC) test kopasanda (*C.odorata* L) leaf methanol extract was actively preventing the growth of *V. harveyi* at minimum inhibitory concentration (MIC) of 0,625 mg/mL. According to Aligiannis *et al.* (2001), MIC below 1.6 mg/ml indicated strong activity. It meant that kopasanda leaf extract at low dose would have strong antibacterial activity. Therefore, kopasanda (*C. odorata* L) plant was a promising candidate to be developed as a naturally active compound source for the vibriosis elimination on prawn.

5. Conclusion

Considering the result of research on phytochemical analysis and antibacterial activity test of kopasanda leaf extract on *Vibrio harveyi*, it was concluded that kopasanda (*C. odorata* L.) leaf was is an effective natural antibacterial because it contained phenolic, steroid, flavonoid, and alkaloid compounds, greatest antibacterial properties and low minimum inhibitory

concentrations suggested strong antibacterial activity kopasanda leaf extract. Kopasanda (*C.odorata* L.) plant as the natural antimicrobial to control disease-causing pathogen, *V. harveyi*, especially in the prawn cultivation.

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