

Isolation and Identification of Bioactive Compound of Kopasanda (*Chromolaena odorata* L.) Leaf to Fight *Vibrio harveyi* on Post-Larval Tiger Prawn (*Penaeus monodon* Fabricius)

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Abstract: It is proved that *Chromolaena odorata* L. is usable natural antibacterial agent to fight *Vibrio harveyi*. The study aims to isolate and identify bioactive compound that is responsible for being natural antibacterial agent to fight *Vibrio harveyi* on post-larval tiger prawn (*Penaeus monodon*). The study has worked to isolate 5 fractions of bioactive compound. Based on the antibacterial activity testing to the four fractions, fraction four is evidently in charge of being antibacterial agent as it has the highest capability than other fractions. Based on the identification of Fraction 4 through FT-IR, UV-Vis and GC-MS methods in comparison to the present literature, the bioactive compound falls within flavonoid compound as it is identified as quercetin.

Key words: Isolation and identification, bioactive compound, *Chromolaena odorata* leaf, *Vibrio harveyi*, tiger prawn (*Penaeus monodon* Fabricius)

INTRODUCTION

Rapid knowledge development on natural product in varied biological activities gives an alternative chance to cope with high mortality rate as the impact of vibriosis disease. The disease is mainly caused by *Vibrio harveyi* to post-larval tiger prawn *P. monodon*. In last years, searching for natural product has been focused on isolation or identification of bioactive compound from the ocean in purpose to fight the vibriosis disease in tiger prawn such as diatomae, sponge, hydrozoan and mangrove. Some species mangrove have been used to fight vibriosis, i.e., species *Avicennia marina* and *Sonneratia caseolaris* (Zulham, 2004). *Halimeda opuntia* collected from the ocean has been reported as natural bacteriostatic and bactericidal against *V. harveyi* that is safe for waters. The extract of a sponge *Geodia* sp. also has antibacterial activity against *V. harveyi* (Isnansetyo *et al.*, 2009), *Padina australis* containing phenolic compound has antibacterial activity against *V. alginoliticus* (Salosso and Jasmanindar, 2014), the extract of methanol from *Euphorbia hirta* has antibacterial activity against *Aeromonas hydrophyla* and *V. harveyi*

(Salosso and Jasmanindar, 2014) and the extract of butanol from *Aaptos aaptos* has antibacterial activity against *V. harveyi* (Rosmiati *et al.*, 2011). One of land plants that lives together with mangrove and can be used as bioactive compound sources is kopasanda (*Chromolaena odorata*) leaf. This plant is used as traditional garden to cure various disease types. The liquid generated by this plant's leaf is usable to treat an injured skin and as blood clotting. The root, then is used as antipyretic and analgesic drug. The flower from this plant can be used to treat an injury and as insecticide.

Empirically, kopasanda has been used, based on a report by some brackishwater farmers in South Sulawesi for shrimp disease control. In fact, the shrimp they cultivate in brackishwater pond stays healthy and presumably, it is carried by the use of kopasanda plant. That is why it needs scientific study.

C. odorata L. is potential to generate bioactive compound as an antibacterial agent. Akinmoladun *et al.* (2007) has reported that the extract of methanol made from *C. odorata* leaf contains flavonoid, tannin, steroid, terpenoid, flavonoid, glycoside and alkaloid. Antibacterial activity testing of kopasanda leaf extract indicates that the

extract can hamper the growth of *V. harveyi* in *in vitro*. It is not only effective to against *V. harveyi* but the compound also designates antibacterial activity against *Bacillus subtilis*, *Stapilococcus aureus* and *Salmonella typhimurium* (Vital and Rivera, 2009). The findings of Harlina suggests that the extract of *C. odorata* L. contains chemical compound component, i.e., flavonoid, saponin, tannin and steroid that allowable to be used as antimicrobial agent. Further, it is proved that the methanol extract of kopasanda leaf is able to obstruct the growth of *V. harveyi* in *in vitro* indicating strong antibacterial activity at which the MIC and MBC values are 0.625 and 1.250 ppm. Based on the toxicity test of *C. odorata* such concentration is safe (nontoxic) for post-larval tiger prawn. The findings of Harlina suggest that bioactive compound of kopasanda leaf is effective if it is used as natural antibacterial agent to fight *V. harveyi* at concentration of 1125 and 1250 ppm. Accordingly, it requires further study to isolate and identify bioactive compound of kopasanda *C. odorata* L. leaf in order to find out type of bioactive compound in responsible to fight *V. harveyi* causing vibriosis disease to post-larval tiger prawn.

MATERIALS AND METHODS

Research site and time: It is carried out in two sites as follows: extraction, isolation and identification of bioactive compound of kopasanda leaf is carried out in Herbal Testing Laboratory, Faculty of Pharmacy, the Hasanuddin University. And, the place where application of active compound in combating vibriosis in tiger prawn is carried out in Field Laboratory and Testing-Brackishwater Pond, Faculty of Fisheries and Marine Science, Muslim University of Indonesia, Makassar. The study is held from April to November, budget year 2015.

Research material and equipments: To make an extract of kopasanda leaf, it uses aquades, pulverized kopasanda leaf and methanol solvent, ethyl acetate or chloroform. Methanol by solvent partitioning is chosen as it gives the best hampering force in antibacterial testing to extract bioactive compound of kopasanda leaf. Research equipments used to extract the leaf are blender, vacuum evaporator, glass jar, stirring rod, measuring cylinder, muslin, and balance. Isolation phase uses Thin Layer Chromatography (TLC) and Chromatographic Column (CC) and a set of UV-Vis, FT-IR and GC-MS instrument to identify bioactive compound of kopasanda leaf.

Research procedures

Collection and extraction of kopasanda by maceration method: Kopasanda leaf-sample collection, processing and extraction. Research sample of kopasanda (*C. odorata*) leaf is collected from testing-brackishwater pond in Brackish Water Cultivation Research and Development Center, Maros-South Sulawesi. Sample processing is carried out by these steps: kopasanda leaf is washed off under flowing water, sliced into small pieces and dried up by herbs dryer at temperature of $>40^{\circ}\text{C}$. Dried-up kopasanda leaf is then pulverized.

Extraction and partition of kopasanda leaf: In extraction step, it uses 500 g pulverized powder of kopasanda leaf which is extracted by maceration (soaking) within methanol solvent for 3×24 h. It is followed by methanol extract and dregs splitting.

Liquid methanol extract of kopasanda leaf is evaporated by vacuum rotary evaporator to take its thick methanol extract. The thick methanol extract and n-hexane solvent are partitioned by liquid-liquid chromatography to get n-hexane extract and residue (water layer). The residue (water layer) is partitioned once again by liquid-liquid partition with ethyl acetate solvent to get ethyl acetate extract and methanol extract.

Isolation of bioactive compound: Isolation of bioactive compound of kopasanda is given to the methanol extract which is generated by partition. It applies two kinds of isolation method, i.e., Thin Layer Chromatography (TLC) and Chromatographic Column (CC).

Thin Layer Chromatography (TLC): In this study, selection of solvent for the isolation process is done by trying some combination to expand the selected extract spot on the TLC. Combined solvent tested in the study is n-hexane/ethyl acetate, ethyl acetate/methanol and methanol/chloroform by multiple sorts of ratio (10:0, 8:2, 6:4, 5:5, 4:6, 2:8, 0:10). 0.02 g active methanol extract is dissolved in 0.5 mL solvent. The extract liquid is then splattered into each plate of silica gel 60 F₂₅₄, 1×10 cm, at the distance of 1 cm from the bottom edge of TLC plate by using capillary tubes. Further, the place is dried up and then eluted by eluent as mentioned above. When expansion motion comes at the boundary mark, elution is stopped. Analytical separated yield by Thin Layer Chromatography (TLC) generates spots where the spots are visible if it is steamed by iodine and UV light at wavelength between 254 nm and 366 nm. Combined solvent that generates the finest spot expansion is used

as eluent to fractionate the selected extract, i.e., active methanol extract, by Thin Layer Chromatography (TLC) and Chromatographic Column (CC).

Fractionation by Chromatographic Column (CC):

Isolation by Chromatographic Column (CC) is carried out when the finest eluent generated by Thin Layer Chromatography (TLC) has been combined. Chromatographic Column (CC) is carried out by holding the column onto perpendicular clamp. Glasswool is applied beneath the column. Before the extract is filled in the column for Chromatographic Column (CC) process, the column is washed off and silica gel solvent is made at first. The 13-15 g silica gel is dissolved in eluent to generate silica gel solvent. When it is done, more eluent is added into the silica gel where the ratio between the eluent added and silica gel solvent is 1:1 (w/v). Silica gel solvent is then filled into the column. Column length is 60 and silica gel length filled into the column is 30-35 cm. When the silica gel solvent has been all filled into the column, it is followed by silica gel saturation within the column. During the process of saturation, aluminium foil is glued on top of column to prevent eluent evaporation to make the silica gel stays wet. The silica gel is saturated for 30-60 min.

The extract fractionated is 0.2 g active methanol extract of kopasanda leaf which will be dissolved in 5 mL methanol pro analysis. Silica gel shall be saturated before the addition of extract. Afterward, when silica gel is saturated, bottom hole plug is opened and let the eluent moves downward. When it reaches upper part of column adsorbant (silica gel must not be dry), shut the hole plug and fill the extract into it. Re-opening the column hole plug is done when all extract is in. Let the extract flows downward to the column adsorbant while keep filling in 5 mL eluent (silica gel must not be dry). The solution flowing out of the column is caught by test tube ± 5 mL on each. Solution in the test tube is then dried up by aerator in fume hood to generate extract residue.

The fraction generated by Chromatographic Column (CC), i.e., extract residue is fractionated once again by Thin Layer Chromatography (TLC). The fraction will be dissolved again in its solvent. Thin layer plate of silica gel 60 F254 is prepared 10 cm in length and 1-2 cm in width. Eluent used herein is same as the eluent used in Chromatographic Column (CC). Fill the eluent into Thin Layer Chromatography (TLC), shut the cover and let it be saturated. Draw a boundary line 1 cm from the edge of each plate.

For the separation by preparative TLC, it uses silica gel 60 F254 plate 10 \times 20 cm. Active fraction is dissolved in

methanol, then it is splattered along the silica gel plate at distance of 1 cm from bottom line and 1 cm from boundary line. The plate is then eluted by methanol/chloroform solvent 8:2. The spots are tape-like form under UV 254 and 366 nm and they are marked by pencil and scraped by spatula tip. Silica consisting of active compound is dissolved in methanol and refined by filter cloth to get pure isolate. Let the pure isolate evaporated in fume hood.

Identification of bioactive compound of kopasanda (*C. odorata* L.) leaf: Identification by Ultraviolet-Visible Spectroscopy (UV-vis).

Take 0.1 active fraction extract which has been concentrated by N₂ and liquefy it into volumetric flask with 10 mL appropriate solvent. Fill the solvent in absorbent cuvette and read the absorbtion using Ultraviolet-Visible Spectroscopy (UV-Vis) which has been first calibrated by suitable blank. The absorbtion is read by wave number 200-400 nm.

Identification by infrared spectroscopy: Identification of pure isolate by Fourier Transform Infrared Spectroscopy (FTIR) is carried out by taking 2 g non-aqueous KBr solid, continued by crushing and pressing it at 2 torr pressure. Drip the Kbr pellet with 1 drop of isolate, analyze it by FT-IR spectrometer between 4000-800 cm⁻¹.

Identification by GC-MS: Active isolate is analyzed by GC-MS. It uses helium as the carrier gas which the flow is set as follows: Temperature of injector is 320°C where initial temperature is 70°C. Temperature elevation rate is 100°C and final oven's temperature is 310°C. When it is done, the outcome of bioactive compound of kopasanda leaf is applied to fight Vibriosis disease in tiger prawn (*P. monodon* Fabr).

RESULTS AND DISCUSSION

Isolation of bioactive compound of kopasanda (*C. odorata* L.) leaf: Based on the antibacterial activity test, bioactive compound containing in some extract types of kopasanda leaf are the strongest. Accodngly, isolation of active compound is given to bioactive compound of kopasanda leaf.

Eluent search by Thin Layer Chromatography (TLC): Thin Layer Chromatography (TLC) is performed in purpose to separate the compound dissolved in bioactive compound of kopasanda leaf. As the outcome, this process is able to separate well between methanol and

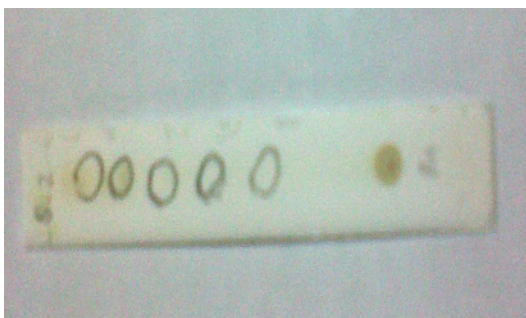


Fig. 1: Result of Thin Layer Chromatography (TLC) to bioactive compound of kopasanda leaf (*C. odorata* L.) Result of partition

Table 1: Inhibition zone diameter (mm) of the five fractions generated by Chromatographic Column (CC) against *V. harveyi*

Types of fraction	Inhibition (mm)±SD zone
1	-
2	-
3	-
4	10

chloroform solvent by ratio 8:2. According to Thin Layer Chromatography (TLC), bioactive compound of methanol extract of kopasanda leaf generated by partition has 5 spots as shown out in Fig. 1.

Fractionation by Chromatographic Column (CC), antibacterial test of bioactive compound generated by fractionation:

Bioactive compound of methanol extract of kopasanda leaf fractionated by Chromatographic Column (CC) in combination with selected solvent as the outcome of the best eluent search by Thin Layer Chromatography (TLC) are methanol/chloroform by ratio 8:2. Bioactive compound of kopasanda leaf filled in the column will move as its molecular weight and every fraction formed in the process is caught by test tube approximately, 5 mL. The extract caught by test tube is distinguished by solvent color flowing out of the column. In Chromatographic Column (CC) process, the extract filled in the column will move as its molecular weight. Chromatographic Column (CC) generates five fractions. The five fractions generated by Chromatographic Column (CC) are tested in antibacterial test to find out which fraction is actively hampering *V. harveyi*. Inhibition zone diameter of the five fractions is made in list in Table 1.

Table 2 displays that the fraction with antibacterial activity against *V. harveyi* is fraction 4 where the inhibition zone 10 mm. Inhibition zone diameter generated by this fraction 4 is narrower than what has been generated by its bioactive compound, i.e., 19 mm. It indicates that the content of compound in bioactive



Fig. 2: Yield of Thin Layer Chromatography (TLC) of fraction 4

compound makes synergy to obstruct the growth of *V. harveyi*. Compound containing in bioactive compound of kopasanda leaf strongly slows down the growth of *V. harveyi* if only the compound stays together where inhibition zone generated by this merger is larger than separated compound. It confirms the findings by Salosso and Jasmanindar (2014) stating that active compound in acetone extract of *Padina auratus* making synergy in slowing down alginoliticus. Based on the antibacterial activity test by Chromatographic Column (CC), fraction 4 is selected in purpose to identify its chemical structure as it may generate inhibition zone diameter compared to other fractions. Fraction 4 is purified in order to identify its bioactive compound by preparative Thin Layer Chromatography (TLC) with the best eluent, i.e., MeOH/chloroform by ratio 8:2. For the separation of fraction 4 by preparative Thin Layer Chromatography (TLC), it uses silica gel 60 F254 plate 10×20 cm. Fraction 4 is dissolved in methanol, then it is splattered along the silica gel plate at distance of 1 cm from bottom line and 1 cm from boundary line. The plate is then eluted by methanol/chloroform solvent 8:2. The spots are tape-like form under UV 254 and 366 nm and they are marked by pencil and scraped by spatula tip. Silica consisting of active compound is dissolved in methanol and refined by filter cloth to get pure isolate. Let the pure isolate evaporated in fume hood. What has been generated by preparative Thin Layer Chromatography (TLC) gets another TLC and Fig. 2 displays a presence of one spot indicating that fraction 4 has been purified.

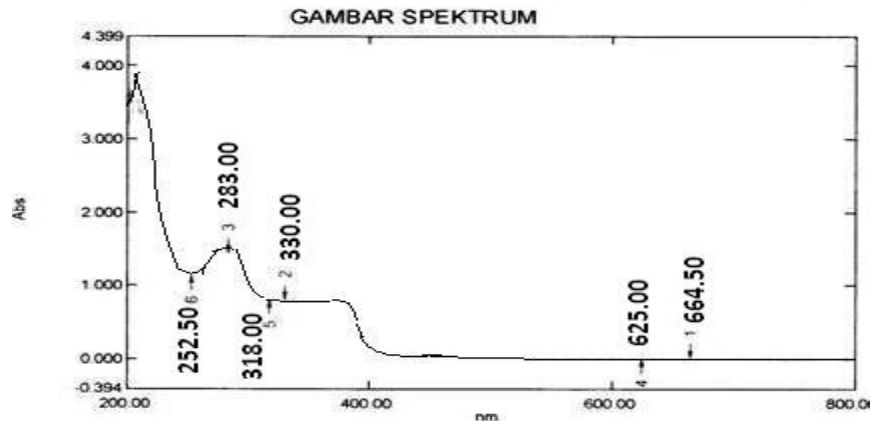


Fig. 3: IR Spectrum of bioactive compound of kopasanda leaf

Table 2: Absorption data of IR spectrum of bioactive compound of Kopasanda (*C. odorata* L.) leaf

Wave Number (cm ⁻¹)	Range (cm ⁻¹)	Intensity	Reference vibration (Silverstein <i>et al.</i> , 2005)
3383	3200-4000	Strong	O-H Stretch
2929	2850-3300	Weak	Alkane C-H Stretch
2855	2850-3300	Weak	Alkane C-H Stretch
1643	1450-1700	Strong	Aromatic C = O
1602	1450-1700	Strong	Aromatic C = C
1020	1020-1080	Medium	Aromatic C-O Stretch

Identification of bioactive compound of kopasanda leaf

The yield of Infrared (IR) spectrum of bioactive compound: FTIR (Fourier Transform Infra Red) is a beneficial instrument to learn about functional group in one compound (Yu *et al.*, 2004). Identification of particular functional group is indicated by specific absorption at particular wave number. It is a product of transition from energy grade (vibration) of every atomic interaction in a molecule, either by bending or stretching. Infrared (IR) spectrum from active fraction (fraction 4) of bioactive compound of kopasanda leaf is drawn as in Fig. 3 and Table 2.

Figure 3 and Table 2 display the presence of hydroxyl (OH) group which is designated by the presence of wide-strong absorption in an area 3383 cm⁻¹. Absorption arising in area 2929 and 2855 cm⁻¹ is an absorption from aliphatic C-H stretch within the molecule compound. And the absorption observed in area 1643 and 1602 cm⁻¹ becomes a proof that there is compound in fraction 4 which has carbonyl group (C = O) and double bond of C = C. Presence of C-C inside the molecule may be seen in absorption appearing in area 1020 cm⁻¹. The five functional groups existing in IR spectrum from fraction 4 are characteristics of flavonoid-type compound.

Ultraviolet spectrometer: Figure 4 displays four maximum wavelength that becomes characteristics of

flavonoid-type compound. In qualitative manner, compound absorption of fraction 4 shows strong absorption at wavelength 252.50; 283; 318 and 330 nm. Ultraviolet spectrum of fraction 4 also shows weak absorption at wavelength 625 and 664.50 nm. Strong absorption at ultraviolet spectrum indicates a presence of many C = C double bonds within aromatic compound of fraction 4. According to Harborne and Williams (2000), phenolic fraction (fraction 4) is presented in Fig. 4. Ultraviolet spectrum of the active compound and their derivatives give strong absorption in area UV spectrum.

Mass spectrum of bioactive compound of kopasanda

(*C. odorata* L.) leaf: Based on mass spectrum output on active sample by GC-MS, it indicates that kopasanda leaf isolate contains chemical compound characterized as follows: there are at least 7 molecular ion fractions with the largest abundance (base peak) where the fragments are molecular ion at m/z 255. Molecular ion breaking from this compound is completely presented in Table 3 and mass spectrum of bioactive compound of kopasanda leaf is shown in Fig. 5.

Figure 5 represents six maximum wavelength spectrums that become characteristics of flavonoid-type compound. In qualitative manner, compound absorption of fraction 4 shows strong absorption at wavelength 252.50; 283; 318 and 330 nm. Ultraviolet spectrum of

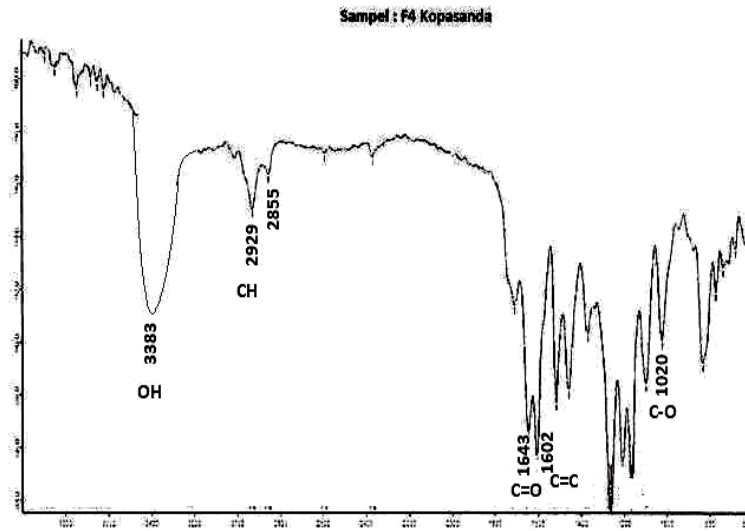


Fig. 4: Ultraviolet spectrum of fraction 4 compound from bioaction compound of kopasanda leaf

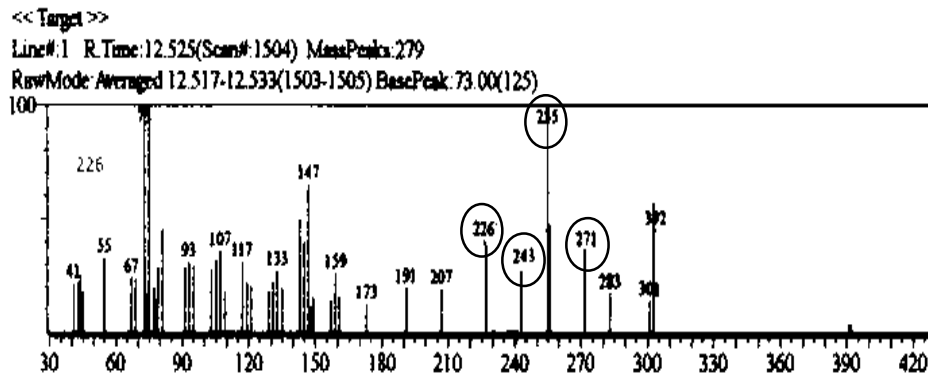
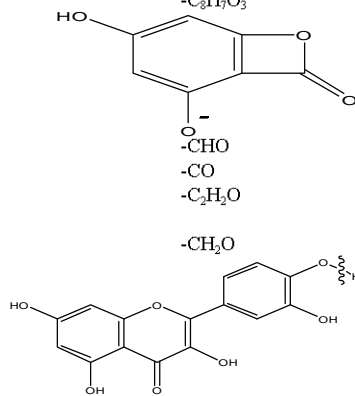


Fig. 5: Analysis of bioactive compounds of kopasanda leaf

Table 3: Molecular ion fraction of flavonoid compound containing in kopasanda (*C. odorata*) leaf

Ion's Mass (M/Z)	Molecular ion fraction
151	-C ₈ H ₅ O ₃
226	-CHO
243	-CO
255	-C ₂ H ₂ O
271	-CH ₂ O
301	-H
302	M+



fraction 4 also shows weak absorption at wavelength 625 and 664.50 nm. Strong absorption at ultraviolet spectrum indicates a presence of many C = C double bonds within aromatic compound of fraction 4. According to Harborne and Baxter (1999), phenolic compound and their derivatives give strong absorption in area UV spectrum.

EIMS Spectrum has also represented a presence of molecular ion at m/z 302 suggesting molecular composition C₁₅H₁₃O₇ (Fig. 5). Molecular ion breaking existing at m/z 271, 255, 243, 226 and 151 happens when molecular ion lose CH₂O, C₂H₂O, CO, CHO and C₈H₅O₃. Based on the analysis result by GC-MS supported by Ultraviolet (UV) spectrum and Infrared (IR) spectrum, it is supposed that the compound isolated from active extract of kopasanda leaf is flavonoid derivatives, broadly known as quercetin (Li *et al.*, 2009) with chemical structure as illustrated in Fig. 6 and 7.

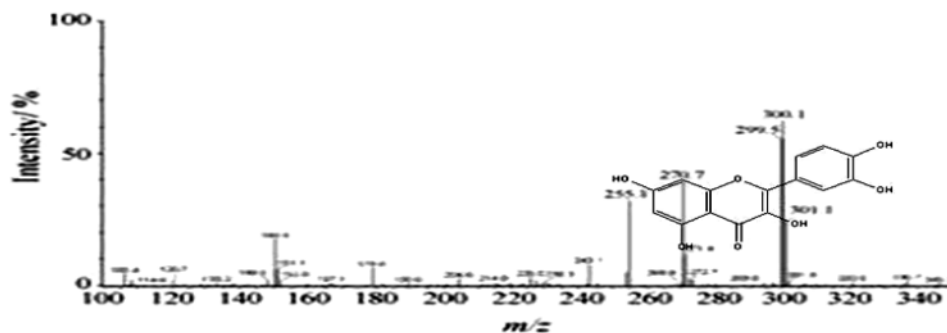


Fig. 6: Mass spectrum of bioactive compound of kopasanda (*C. odorata*) leaf (top) mass spectrum of comparator (bottom) (Li *et al.*, 2009)

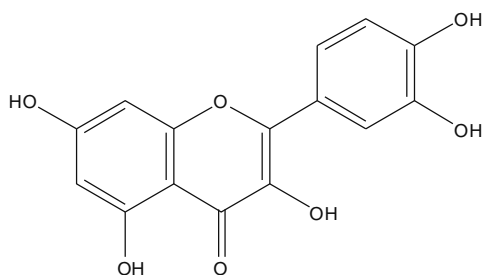


Fig. 7: Quercetin (Li *et al.*, 2009)

Bioactive compound found in kopasanda leaf is flavonoid compound. Ability of flavonoid compound as antibacterial agent against *V. harveyi* is highly affected by its biological activity. Further explanation suggests that if the bioactive compound has contact with *V. harveyi* bacteria, it will react with amino acid compounds to arrange bacterial cell wall and also bacterial DNA that mainly composes the nucleus cell as information and administrative center of the cell. This reaction happens when a base compound chemically reacts an acid compound; amino acid in this case. This reaction makes structural and composition changes of the amino acid since most of amino acid has reacted base group of alcaloid compound. Amino acid composition change will obviously change DNA strand of the nucleus cell that initially has acid-base pairing composition. Amino acid strand change in DNA will change genetic equilibrium in DNA acidity causing damage on the DNA of *V. harveyi*.

As mentioned above that the methanol extract from kopasanda leaves contain flavonoid compound and some medicinal herb extracts contain flavonoid show the antimicrobial activities (Waage and Hedin, 1985; the flavanoid antibacterial activities to *Staphylococcus epidermidis* (Vaughn, 1995; Shutz *et al.*, 1995; Colombo and Bosisio, 1996); flavonoid compound from *Tagetes minuta* leaf show its

antibacterial activities to either positive or negative gram bacteria (Tereschuk *et al.*, 1997). According to Cowan, flavonoid is a group of phenolic derivative compound which is against the microbe, insect and herbivore.

Furthermore, Cody (1987) reported that flavonoid shows important biological activities used in bacteriology, pharmacology and medication, due to its bactericidal nature. The existence of Hydroxyl (OH) chain in flavonoid compound will intensify its antibacterial activity (Alcaraz *et al.*, 2000). Besides the existence carbonic chain (C = O) in flavonoid compound also affect the antibacterial activity through the interaction with some enzims, usually with hydrogen chain (Cotelle *et al.*, 1996).

CONCLUSION

From kopasanda leaf, the study has successfully isolated the bioactive compound which has antibacterial activity identified as the derivative of flavonoid compound or known as quercetin which is responsible for safer and more effective *V. harveyi* antibacterial activity on tiger prawn larva.

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