IN VITRO ANTI-VIBRIO ACTIVITY OF MIANA LEAVES (*Coleus scutellarioides* (L) Benth)

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Abstract

The aim of this research is to determine the bioactive compounds of miana leaves of Coleus scutellarioides (L) Benth against Vibrio spp.. This research was carried out from April to August 2018 at the Fish Pests and Diseases Laboratory, Department of Fisheries, Gadjah Mada University. Miana leaf samples were dried at room temperature, macerated with ethanol solvent and tested for antibacterial activity. Separation of bioactive compounds was carried out using liquid partition and silica gel using the column chromatography method. MIC (minimum inhibitory concentration) is applied to evaluate antibacterial activity. The results showed that the highest antibacterial activity was found in the chloroform fraction with inhibitory zone diameters for V. parahaemolitycus, V. alginolitycus, and V. harveyii of 25 mm, 26 mm, and 20 mm at a dose of 20 µg/disk. These findings indicate that the antibacterial activity of miana leaf crude extract is comparable to oxytetracycline with inhibition zones of 25 mm, 31 mm and 20 mm respectively against similar bacterial strains at 20 µg/disk. Therefore, miana leaves are a prospective natural source of anti-vibrio compounds.

Key Words: Antibacterial Activity Test, Chloroform Fraction, Inhibition Zone, Vibrio

INTRODUCTION.

Cultivated shrimp production in Indonesia has a tendency to decline. Unsupportive environmental quality, cultivation management, and inadequate biosecurity systems are the main supporting factors for the decline in production due to crop failure. These three factors trigger the emergence of disease outbreaks that cause high mortality rate [1] and causes a drastic reduction in production.

Vibrio spp. is considered the most lethal pathogen in shrimp aquaculture [2][3]. *Vibrio* spp. massively infects aquaculture facilities and leads to significant financial losses [4]. Several vibriosis prevention and controlling methods have been conducted through the administration of antibiotics, vaccines, and probiotics [5]. However, the use of antibiotics is not recommended due to a potential strengthening in bacteria resistance. Therefore, the use of natural bioactive compounds is highly recommended.

Several secondary metabolite form derived from plants can be used as a natural antibacterial agent. In order to confirm the bioactive compound of plants, phytochemical testing is required. In general, plants contain bioactive matter such as flavonoids, tannins, and saponins. Tannins are able to inhibit the microbes' growth by constraining the enzymes such as cellulose, pectinase, oxidative peroxide [6] In addition, phenols compounds can be toxic to bacteria in high concentrations. Phenol is also known as carbolic acid which is able to eliminate germs [6].

Miana leaves are rich in secondary metabolite compounds in the form of flavonoids, steroids and tannins [7]. Studies on miana leaves against certain pathogens have been documented, including anthelmintic effects on tapeworms in chickens [7], anticestodal effects on worms in mice [8], and mycobacterium tuberculosis in mice [9]. Research on anti-vibrio activity has been carried out [10][11][12][13][14]. However, in vitro testing of miana leaves as an anti-vibrio compound has not been found. Therefore, this research is considered important to determine the ability of Miana leaf extract to inhibit the growth of Vibrio sp. in vitro

Article history:
Diterima / *Received 26 December 2022*
Disetujui / *Accepted 21 March 2024*
Diterbitkan / *Published 30 March 2024*

METHOD

The miana leaves used are from the purple miana plant (*Coleus scutellarioides* L Benth) originating from South Sulawesi, Indonesia. Miana leaves are deep purple or blackish purple. The edges of the leaves are serrated with a pattern of crossed lines on the leaf surface and are finely hairy.

The research was carried out from April to August 2018 at the Faculty of Hydrology and Fish Health Management, Department of Fisheries, Faculty of Agriculture, Gadjah Mada University and the Laboratory of the Balik Diwa Marine Technology College, Makassar.

Natural bioactive compound and vibrio spp.

The miana leaves *Coleus scutellarioides* (L) Benth) were used as natural bioactive compound. The *Vibrio* spp.. bacteria including *V. harveyii* SP 25, *V. alginoylticus* GD 22 and *V. parahaemolyticus* GD 36 were taken from isolated bacteria collections at the Department of Fisheries, Faculty of Agriculture, at Gadjah Mada University.

Extraction Procedure.

The maceration method is applied to the extraction process. Miana leaves are first dried and ground into flour before being extracted. Ethanol (96%) was used for extraction, because ethanol is a versatile polar solvent and is very good for use preliminary extraction [15] as well as its ability to withdraw active compounds more quickly [16]. simplicia mixed with ethanol in a ratio of 1:5 which takes 10 minutes for homogeneity and is stored for 24 hours, and the process was repeated three times. Simplicia extracts were mixed and concentrated using a rotary evaporator at a temperature of 40˚C [17]. The concentrated extract was stored in a refrigerator for further testing.

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Partition of bioactive compounds (separation of polar, semi-polar and non-polar compounds)

Testing for semi-polar and non-polar compounds was carried out to determine groups of compounds that have activity against Vibrio sp., using a method called partitioning. Dissolution of compound groups through ethanol (polar), hexane (non-polar) and chloroform (semi-polar) fractions. Method based on Hamdillah, et al (2019)[17], ethanol extract (crude extract) produced from maceration hexane solvent was added (ratio 1:1). Shaking was carried out for 10 minutes to separate the polar and non-polar fractions. Fractions are separated in different places. Ethanol (polar fraction) is added to distilled water 1:1, shaken until homogeneous. Separating polar and semi-polar compounds in a separating funnel, chloroform was added in a 1:1 ratio and shaking was carried out for 5 to 10 minutes. All partitions done twice. The partition extract of each fraction was evaporated and stored in a refrigerator for further testing..

Test Media and Bacteria Preparation

Solid and liquid Zobell was used as a medium to culture the bacteria using yeast extract (Oxoid, the United Kingdom), Kaliumbromida (Germany), bacto agar (Oxoid, the United Kingdom), and bacteriological peptone (Oxoid, the United Kingdom). Mueller Hinton Broth (Laboratorios Conda) was used for the minimum inhibitory concentration (MIC) test. A total of 4 grams of bacto agar medium was mixed with 100 mL of distilled water then heated with an electric stove to homogenize the media. The media was autoclaved for between 15 and 20 minutes at a temperature of 120ºC. The media was divided into several petri dishes. The Vibrio spp.. bacteria test was a subculture to the Zobell broth medium and then incubated for 24 hours at 30 ºC. The bacteria were transferred to the Zobell broth to be cultured in the media.

Anti-Vibriosis Activity Test

The diffusion method on double layer agar was used to test the antivibrio test [18]. Prepared petri dish with solid media was added 5-10 mL Zobel soft which contained active bacteria. The disk was prepared by placing it in a petri dish which had been labeled according to the extract to be tested. A total of 20 µg of extract was added to each disk at a concentration of 500 µg per mL and 1000 µg per mL. The paper discs were air dried and placed on the surface of the media and

incubated at 30 ºC for 24 hours. The response to the potential antibacterial was determined by observing the formation of a clear zone around the paper disc which known as the zone of inhibition. Clear zone diameters were measured using calipers in millimeters.

Thin-layer chromatography (TLC).

Thin-layer chromatography was applied for separating the sample based on the polar component [19]. The retention factor is the quotient of distance of substance per distance of solvent and thus calculated using the following equation:

$$Rf = \frac{\text{Distance of substance}}{\text{Distance of solvent}}$$

Extracts can be tested for polarity via thin layer chromatography (TLC). The TLC test was carried out by cutting a TLC plate measuring 10 x 1.5 cm. The extract to be analyzed is dissolved until homogeneous. The solution is applied to the bottom border of the TLC plate which has been determined at a distance from the bottom of the TLC plate. The plate is then placed vertically into a closed tank containing the eluted solvent. Please note that the bottom edge of the plate must be submerged in the solvent, but the compound concentration must be above the eluent (solvent) limit. After propagation occurs there will be two components that interact with the adsorbent and interact strongly with the eluent. Both components can be identified by removing the plate from the tank until it is dry from the solvent [20][21]

Minimum Inhibitory Concentration (MIC).

The MIC test was performed using the 96-well microdilution method [22][23][24][18]. A total of 500 µg/mL and 1000 µg/mL of natural bioactive concentration were used. There were two different controls: negative control (without extract and antibiotic) and positive control (using the commercial antibiotic, oxytetracyclin). The treatment was replicated twice. The MIC test was qualitatively observed by observing the colour change in the medium after resazurin solvent was dropped into the medium. Blue colouring indicated no bacteria growth and pink colouring indicated bacteria growth after 24 h at 30 ºC of incubation.

Data Analysis

The data was analyzed descriptively comparatively and presented in the form of tables and figures

RESULTS AND DISCUSSION.

The findings of this study are illustrated in Table 1. Table 1 describes the activity of antibacterial using miana leaves extract derived from three fractions including ethanol, chloroform, and hexane.

Table 1 illustrates that bacterial inhibition occurred only in the chloroform fraction extract, with each inhibition value at a concentration of 500 µg per disk and 1000 µg per disk. The zone of inhibition of chloroform fraction extract is characterized by the formation of a clear zone around the disc paper on solid zobell media which is clearly seen in Figure 1 below.

Table 1. The inhibitor power of three different fractions on three different bacteria

No	Bacteria	Fractions	Inhibitor power (mm)		
			500 µg/disk	1000 µg/disk	Control
1	*V. parahaemolitycus*	Hexane	-	-	-
		Chloroform	12	23	25
		Ethanol	-	-	-
2	*V. alginolitycus*	Hexane	-	-	-
		Chloroform	17	28	31
		Ethanol	-	-	-
3	*V. harveyii*	Hexane	-	-	-
		Chloroform	15	20	21
		Ethanol	-	-	-

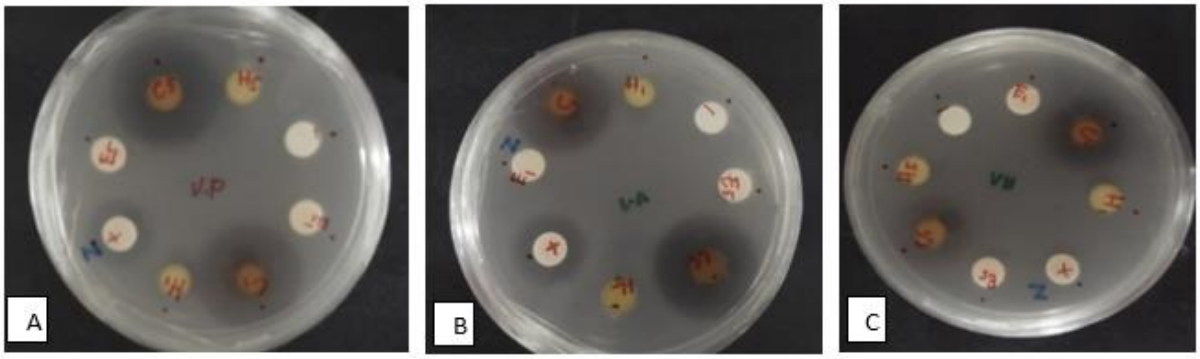

Figure 1. The antibacterial activity of crude miana leaves extract : (A) Diameter inhibitor of *V.parahaemolitycus* GD 36, Chloroform, (21 mm and 25 mm), oxytetracyclin (16 mm); (B) Diamater inhibitor of *V. alginolitycus* GD 22, Chloroform (23 mm and 26 mm), oxytetracyclin (15 mm), (C) Diameter inhibitor of *V. harveyi* SP. 25, Chloroform (14 mm and 20 mm). Oxytetracyclin (9 mm).

The extract obtained from chloroform fraction indicates inhibitory activity against GD 22 Vibrio spp. (Table 1). This chloroform fraction is known as a non-polar solvent which is able to dissolve several compounds contained in miana leaves. The chloroform can dissolve the steroids and small amounts of flavonoids and triterpenoids [7]. In addition, flavonoids are the largest phenol group found in nature [25]. The active ingredient of flavonoids is an important component in building the immune
system because it boosts lymphocyte proliferation. Flavonoids can also perform as immunomodulators which increase lymphocyte proliferation activity [26][27][28]. Phenol is germicidal at high concentrations leading to coagulation and protein precipitation whereas at low concentrations it causes protein denaturation without coagulation. Phenols can also stimulate the central nervous system (CNS) and cause paralysis due to muscle spasms [29]. Phenols can be easily absorbed through tissues and even skin. It can also be absorbed through blood flow and excretes through urine.

The presence of natural steroids or plant sterols that have long been identified are campesterol, stigmasterol, and β-sitosterol and also known as ingredients that have anti-carcinogenic, antioxidant, hypoglycaemic effects [30][31][32]. They significantly contribute to hepatoprotection effects. Nonetheless, in terms of their bacteria activity inhibitor, they work through interference with the mechanism of action of the bacterial cells that can lead to death.

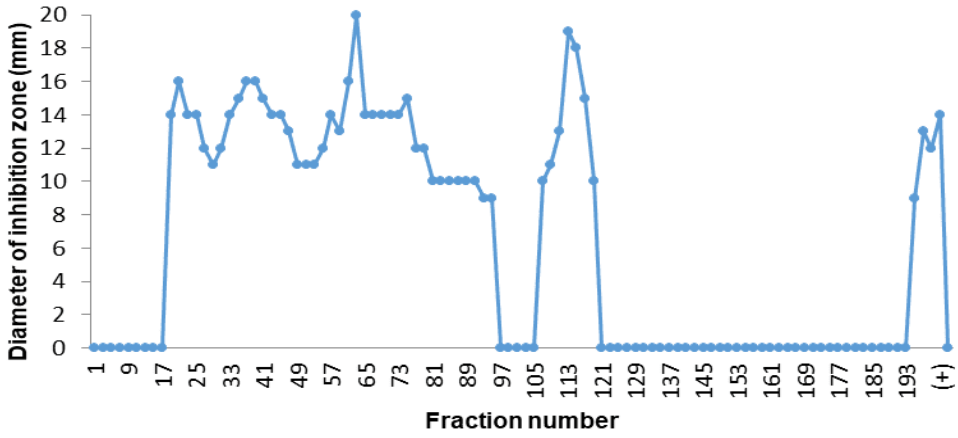

Figure 2. The antibacterial activity fraction of extract miana obtained from the chromatography column

The chloroform fraction of miana leaf extract can inhibit the growth of Vibrio sp. which is comparable to the inhibitory value of the antibiotic oxytetracycline. The inhibitory power value of the chloroform fraction on V. parahaemolyticus was 21-25 mm, V. alginolyticus 23-26 mm, and V.harveyi 14-20 mm, comparable to the inhibition value demonstrated by the antibiotic oxytetracycline of 20-31 mm. Each extract is 20 µg per disk. Therefore, miana leaves can act as an alternative natural ingredient to replace antibiotics to inhibit the growth of the bacteria V. parahaemolitycus GD 36, V. alginolitycus GD 22, and V. harveyii SP 25.

The antibacterial activity of miana leaf extract is still stronger than that of dragon scale leaves which produces activity (inhibitory power) of 14.8 mm against V. harveyi and 11.1 mm against V. parahaemolitycus with an incubation period of 48 hours [33]. Likewise with the antibacterial activity of kitolod leaves, with inhibition against V. harveyi (5.5 mm), V. parahaemolyticus (5.83 mm), and V. algynolyticus (7.33 mm) [34]. which is weak and very low compared to the inhibiting activity of Miana leaves. Likewise, Euchema cottonii extract tested against V. parahaemolyticus, V. Alginolyticus, and V. charcariae has a the highest barrier against V. parahaemolyticus was 24.1 mm [35].

The active fraction is grouped based on antibacterial activity, and about 27% of the fractions have high antivibrio activity found in the chromatography column (Figure 2). A total of 50% fraction in the antivibrio activity is considered to closely related with the active compound content of the miana leaves in the form of tannin and saponin which are quite high, as well as flavonoids, terpenoids and tannin [7][36]. These bioactive compounds are widely known to have the The active fraction is grouped based on antibacterial activity, and about 27% of the fractions have high antivibrio activity found in the chromatography column (Figure 2). A total of 50% fraction in the antivibrio activity is considered to closely related with the active compound content of the miana leaves in the form of tannin and saponin which are quite high, as well as flavonoids, terpenoids and tannin [7][36]. These bioactive compounds are widely known to have the ability as an antibacterial and antiviral through evaluation and research in overcoming diseases. Many people have been using miana leaves as traditional medicine. Miana leaves are believed to cure several diseases, such as coughing, influenza, and spots on the lungs, ulcers, hemorrhoids, and diabetes. According to Lisdawati et al. (2008)[36], miana leaves contain terpenoids, large amounts of tannin, condensed tannin, and flavonoids. Miana is also one of the plants included in the list of 66 biopharmaca plant commodities based on the Decree of the Minister of Agriculture No. 511/Kpts/PD.310/9/2006 [37].

***Minimum Inhibitory Concentration* (MIC).**

The results of the minimum inhibitory concentration (MIC) test showed the inhibitory value of miana leaf extract against Vibrio sp. (GD 22) at a concentration of 500 µg / mL. This MIC test was conducted after the extracting process through screening using preparative chromatography and TLC columns. The inhibitory values of the concentrations are still categorized as high concentrations compared to the value of commercial antibiotic concentrations which have been able to inhibit the concentration of 10 µg per mL in the same bacterial strain, such as GD 22. This finding confirmed that the bonding or absorbability of the compound fraction is affected by the presence of the silica. Silica has a silanol group and the free hydroxyl contained in which capable of binding to compounds and forms strong hydrogen bonds [38]. The stronger the hydrogen bond is formed, the stronger the compound is retained by silica. Thus, it affects the activity of the active compound incorporated in the extract.

CONCLUSION

Miana leaves are able to inhibit the growth of Vibrio sp. in in vitro conditions, with the same strong inhibition as the antibiotic oxytetracyclin. These findings indicate that miana leaves are recommended for use in treating Vibrio spp. In vivo testing is needed for further testing to determine the effectiveness of using Miana leaves in preventing Vibrio sp bacterial infections.

ACKNOWLEDGEMENT

We would like to thank the Directorate General of Technology Research and Higher Education for sponsoring this research through the University Collaborative Research Funding (PKPT) program in 2018.
We also thank the Chancellor of the Balik Diwa Maritime Business and Technology Institute, who provided support for the research conducted by the author.

REFERENCES

[1] FAO. 2014 The state of world fisheries and aquaculture: opportunities and challenges. FAO Fisheries and Aquaculture Departement. Food and Agriculture Organization of the United Nations. Rome

[2] Austin, B., and D.A. Austin. 2007. Bacterial fish pathogens: disease of farmed and wild fish, 4 th Ed. Springer Praxis Publishing, Chichester, United Kingdom, 552 pp

[3] Feliatra, Zainurinand Dessy Yoswaty, 2014. Phatogenity of Vibrio sp on Tiger Prawn (Penaeus monodon). 1 (2). 23-36 [in Indonesian]

[4] Wei, Q., 2002. Social and economic impacts of aquatic animal health problems in aquaculture in China. In: Arthur JR, Philips MJ, Subasinghe RP, Reantaso MB, and MacRae IH. (Eds). Primary Aquatic Animal Health Care in Rural, Small-Scale, Aquaculture Development. FAO Fish Technical Paper No. 406. FAO. 55-61pp

[5] Dhayanithi, N.B., Ajith Kumar., T.T., Arockiaraj, J., Balasundaram, C., Harikrishnan, R., 2015. Dietary supplementation of Avicennia marina extract on immune protection and disease resistance in Amphiprion against Vibrio alginolyticus. *Fish & Shelfish Immunology*. 45, 52-58

[6] Rosyidah, Cicik., 2008 Tamarine *Tamarindus Indica L* as biocoagulant on water quality from physical chemical, and bacteriology. Thesis, School of Postgraduate, Department of Biology, Faculty of Science and Technology. Malang: Universitas Islam Negeri Malang. [in Bahasa Indonesia]

[7] Ridwan Y., Darusman K.L., Satrija F., Andaryani E., 2006 the chemical compound of painted nettle (*Coleus blumei* Benth) leave extract and its anthelmintic activity against chicken tapeworm. Jurnal III Pertanian Indonesia. 11 (2) : 1-6 [in Bahasa Indonesia]

[8] Ridwan Y., 2010 Anticestoda activity on miana leaves *Coleus blumei* Benth on *Hymenolepis microstoma* taken from mice. Dissertation. School of Postgraduate, Bogor Agricultural University. Bogor. [in Bahasa Indonesia]

[9] Palette T., 2017. Effects of Miana Leaves (*Coleus Scutellarioides* (L) Benth) Extract on M*ycobacterium* Tuberculosis in inducted Mus musculus using Mycobacterium under *intraperitonium* condition. Dissertation. School of Postgraduate of Medical Science. Universitas Hasanuddin. Makassar.

[10] Saptiani G., Prayitno S.B., and Anggoro S., 2013 Antibacterial extract derived from *Acanthus Ilicifolius* on vibrio Harveyi In Vitro [Potensi Antibakteri Ekstrak Daun Jeruju (Acanthus ilicifolius) Terhadap *Vibrio harveyi* Secara In Vitro]. *Jurnal Kedokteran Hewan*. 7,(1):17-21 [in Bahasa Indonesia]

[11] Purnama R., Melki EP W.A., and Rozirwan, 2010 Potensial of Halimedha renchii Seaweed Extract as Antibacterial Vibrio sp. [Potensi Ekstrak Rumput Laut Halimedha rencii sebagai Antibakteri Vibrio sp.]. *Maspari Jurnal* 02 (2011) 82-88.

[12] Hidhayati N., 2017 Extraction, Fractions, and Characterization of Antibacterial Compound derived from Mangrove Leaves *Ceriops Decandra* (Griff.) Ding

Hou. Thesis. School of Postgraduate in Universitas Gadjah Mada. Yogyakarta. 65p [in Bahasa Indonesia]

[13] Azis, 2019 Antibacterial Analysis of Dragon Scales Leaves under invitro condition (*Drymoglossum Pilosellaoides*) on *Vibrio harveyi* and *Vibrio parahaemolyticus*. Journal of Aquaculture and Fish Health. 8 (2). 86-91 [in Indonesian]

[14] Hamdillah A., Isnansetyo A., Istiqomah I., Puspita I.D., Handayani D.P., dan Kaneko T., 2019 Antibacterial Activity of Coastal Plants and Marine Sponges from Kei Island Indonesia against Bacterial Fish Pathogens. *Pharmacognosy Journal*, 11 (4), 812-817

[15] Harbone J B. 1987. Phytochemical Methods, A Guide to Modern Ways of Analyzing Plants. Translation. K. Padmawinata and Soediro. Publisher: ITB. Bandung.

[16] Tiwari P., Kumar B., Kaur M. Kaur G., Kaur H., 2011. Phytochemical screening and Extraction: A Review. Internationale Pharmaceutica Sciencia, Vol. 1, Issue 1; 98-106.

[17] Hamdillah A., Isnansetyo A., Istiqomah I., Puspita I.D., Handayani D.P., Kaneko T., 2019. Antibacterial Activity of Coastal Plants and Marine Sponges from Kei Island Indonesia against Bacterial Fish Pathogens. Pharmacogn Journal; 11(4):812-817.

[18] Isnansetyo A., Kamei Y., 2009 Anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activity of MC21-B,an antibacterial compound produced by the marine bacterium *Pseudoalteromonas phenolica* O-BC30T. *International Journal of Antimicrobial Agents*. 34 (2) : 131-135

[19] Wall P.E., 2005 Thin-layer chromatography a modern practical approach. The Royal Society of Chemistry. 176p

[20] Rosamah E., 2019. Thin layer chromatography, a simple method for the chemical analysis of woody plants. Mulawarman University Press. 69p.

[21] Cahyono B., Suzery M., 2020. Natural material separation methods (Theoretical and experimental aspects). Kompas Ilmu, 117p.

[22] Horikawa M., T. Noro, Y. Kamei, 1999. In vitro anti-methicillin resistant *Staphylococcus aureus* activity found in extract of marina algae indigenous to the coastline of Japan. *J. Antibiotics*. 52(2):186-9

[23] Miller R.A., Walker R.D., Baya A, Clemens K., Coles M., 2003. Antimicrobial Susceptibility Testing of Aquatic Bacteria: Quality control Disk Diffusion Ranges for Escherichia coli ATCC 25922 and *Aeromonas Salmonicida* Subsp Salmonicida ATCC 33658 at 22 and 28 ^{0}C. *J. Clin. Microbiol*. 41:4318-4323

[24] Isnansetyo A., Kamei Y., 2005. Direct antagonistic Method for screening anti-methicillin resistant *Staphylococcus aureus* (MRSA) substance, producing marine bacteria. *Biota*. 10 (3): 141-145

[25] Achmad, S.A., 1986 Organic Chemical from Nature 1 [Kimia Organik dari Alam 1]. Penerbit Karunika Jakarta, Universitas Terbuka. 148p [in Indonesian]

[26] Meiriana Y., 2006 Effects of Red Fruit (Pandanus conoideus Lam) on Proliferation of Human Lymphocyte Cell under invitro condition, Thesis, School of Postgraduate of Institut Pertanian Bogor. [in Indonesian]

[27] Wiedosari E. 2007 Role of Natural Immunomodulator (Aloe Vera) in Cellular and Humoral Immune Responses. Indonesian Bulletin of Animal and Veterinary Sciences. 17 (4): 165-171 ;in Bahasa Indonesia]

[28] Wahyuniarti I., Soesatyo M.H.N.E., Ghufron M., Yustina, Sumiwi A.A., Wiryawa S., 2009 Red fruit oil increases the lymphocyte proliferation activity of mice after infection Listeria Monocytogeneses [Minyak Buah Merah Meningkatkan Aktivitas Proliferasi Limfosit Limpa Mencit Setelah Infeksi Listeria Monocytogenesis]. Jurnal veteriner, 10 (3) : 143-149 [in Bahasa Indonesia]

[29] Goodman, L. S. and Gilman, A. 1960. The Pharmacological Basis of Therapeutics. New York: MacMillan Company. 1831p.

[30] Choi, J.M., Lee, E.O., Lee, H,Y., 2007 Identification of campesterol from *Chrysanthemum coronarium* L. and its antiangiogenic activities. *Phytother Res*. 21, 954-959

[31] Panda, S., Jafri, M., Kar, A., and Meheta, B.K., 2009 Thyroid inhibitory, antiperoxidative and hypoglycemic effects of stigmasterol isolated from Butea monosperma. Fitoterapia, 80(2):123-680

[32] Gabay, O., Sanchez, C., Salvat, C., 2010 Stigmasterol: a phytosterol with potential anti osteoarthritic properties. *Am J Clin Nutr*, 18, 106-116.

[33] Azis, 2019. In Vitro Analysis of Dragon Scale Leaves (Drymoglossum pilosellaoides) Antibacterial Activity Against Vibrio harveyi and Vibrio parahaemolyticus Bacteria. Journal of Aquaculture and Fish Health Vol. 8(2);86-91.

[34] Hidayati S., Lumbessy S.Y., dan Azhar F., 2021. Antibacterial Activity of Kitolod Leaves Ethanol Extract (Isotoma longiflora) Against Bacteria Vibrio sp. Reason Vibriosis In Vaname Shrimp (Litopenaeus vannamei). Bioscientist : biological scientific journal. Vol. 9(1);86-95.

[35] Purnama R., Melki, Putri W.A.E., Rozirwan, 2011. Potential of Halimeda renchii and Euchema cottonii Seaweed Extracts As an Antibacterial Vibrio sp. Maspari Journal, 02;82-88.

[36] Lisdawati V., Mutiatikum D., Alegantina S., Astuti Y.N., 2008 Characterization of miana leaves *(Plectranthus Scutellarioides* (L.) Bth.), and piper betle L.) Using physical and chemical obtained from Locally Ingredients for Anti-malaria North Sulawesi. (4) 18, 213-225

[37] Promosiana, A., N. Indartiyah, and M. P Tahir. 2007 Mapping on Bioregional Biopharmacy plant [Peta Potensi. Bioregional Tanaman Biofarmaka]. Direktorat Budidaya Tanaman Sayuran dan Biofarmaka, Dirjen Hortikultura, Deptan RI. Jakarta 73-91pp [in Bahasa Indonesia]

[38] Salituro, G.M. and Dufresne, C., 1998 Isolation by Low Pressure Column Chromatography, In R. J. P. Cannell, ed. *Methods in Biotechnology Vol 4, Natural Products Isolation*, Totowa, New Jersey: Humana Press Inc.