

## IMMUNOSTIMULATORY EFFECT OF FATTY ACID FROM STARFISH (*Acanthaster planci*) ON LYMPHOCYTE PROLIFERATION *IN-VITRO*

### *Efek Imunostimulator Asam Lemak dari Bintang Laut (Acanthaster planci) terhadap Proliferasi Limfosit Secara In-Vitro*

M. Janib Achmad<sup>1,2\*</sup>, Alim Isnansetyo<sup>2</sup>, Noer Kasanah<sup>2</sup>, Ustadi<sup>2</sup>, and Kamiso<sup>2</sup>

<sup>1</sup>Faculty of Fisheries and Marine Science, Khairun University, Ternate, Jl. Pertamina, Kampus II Gambesi, Ternate, North Moluccas, Indonesia

<sup>2</sup>Department of Fisheries, Gadjah Mada University, Yogyakarta, Jl. Agro bulak sumur, Yogyakarta, Indonesia

\*Correspondence Author: [mjachmad@yahoo.com](mailto:mjachmad@yahoo.com)

Article history:

Received: 18 August 2014; Revised: 2 September 2014; Accepted: 16 Oktober 2014

#### ABSTRACT

The aims of this study were to investigate lymphocyte proliferation activity and to identify chemical constituents of active fractions of starfish *Acanthaster planci*. *Acanthaster planci* was collected from Ternate Island, North Moluccas, extracted with distilled methanol and water, partitioned with gradient chloroform-hexane-methanol-water and fractionated in column chromatography using silica gel and hexane-ethyl acetate-methanol. The active compound had been purified by Thin Layer Chromatography (TLC) and identified by Gas Chromatography-Mass Spectrometry (GC-MS). The lymphocyte proliferation activity was measured based on % Stimulation Index (SI) from sample absorbency and control absorbency. The result showed that the 3 fractions of hexane fraction exhibited lymphocyte proliferation activity. Fraction 1 was able to increase lymphocyte proliferation at 48 hours and 72 hours by 55% (80 µl/ml) and 88% (160 µl/ml) increase, and fraction 2 had 77% (160 µl/ml) and 86% (640 µl/ml) increase. Meanwhile, fraction 3 had 75% (640 µl/ml) and 89% (640 µl/ml) increase. Metabolite analysis of active fraction using GC-MS yielded a number of chemical constituents that was dominated by fatty acid. The study concluded that starfish *A. planci* from Ternate Island has a potential source of immunostimulator.

**Keywords:** *Acanthaster planci*, immunostimulatory, lymphocyte proliferation

#### ABSTRAK

Penelitian ini bertujuan menginvestigasi aktivitas proliferasi limfosit dan mengidentifikasi senyawa kimia dari fraksi aktif bintang laut *Acanthaster planci*. *Acanthaster planci* diambil dari pulau Ternate, Maluku Utara, yang diekstraksi dengan air dan metanol, dipartisi dengan gradien kloroform-heksan-metanol-air dan fraksinasi pada kolom kromatografi menggunakan *silica gel* dan gradien heksan-etil asetat. Senyawa aktif dipurifikasi dengan Kromatografi Lapis Tipis (KLT) dan diidentifikasi dengan *Gas Chromatography-Mass Spectrometry* (GC-MS). Aktivitas proliferasi limfosit diukur berdasarkan % Stimulasi Indeks (SI) dari absorbansi sampel dan absorbansi kontrol. Hasil menunjukkan 3 fraksi dari fraksi heksan memperlihatkan aktivitas proliferasi limfosit. Fraksi 1 dapat meningkatkan proliferasi limfosit pada 48 jam dan 72 jam dengan peningkatan 55% (80 µl/ml) dan 88% (160 µl/ml) dan fraksi 2 dengan peningkatan 77% (160 µl/ml) dan 86% (640 µl/ml). Sementara itu, fraksi 3 dengan peningkatan 75% (160 µl/ml) dan 89% (640 µl/ml). Analisis senyawa metabolit dari fraksi aktif dengan GC-MS menghasilkan unsur-unsur kimia yang didominasi asam lemak. Penelitian ini menyimpulkan bahwa bintang laut *A. planci* dari pulau Ternate merupakan sumber daya yang berpotensi sebagai imunostimulator.

**Kata Kunci:** *Acanthaster planci*, imunostimulator, proliferasi limfosit

Permalink/DOI: <http://10.15578/squalen.v9i3.109>

#### 1. Introduction

Marine biodiversity resources are reservoir compounds of active chemicals which are beneficial as compound guides in discovering drugs. Particularly, *Acanthaster planci* is one of those significant biological

resources. *Acanthaster planci* is a starfish from class *Stelleroidea*, family *Acanthasteridae* which is covered by poisoning thorn (Fraser et al., 2000). The starfish is the main coral's predator which lives in a lagoon or in 3-10 meters depth in coral reefs area. As the main feed of *A. planci* is coral, it is estimated that the

existence of the starfish would balance the development of coral reefs. Unfortunately, large quantity of *A. planici* would also be responsible for the coral reefs devastation (Sapp, 1999; Houk et al., 2007; Mendonca et al., 2010).

Previous studies reported that *A. planici* has important chemical compounds as primary and secondary metabolites such as uracil deoxyribosidase, unsaturated fatty acid, polyunsaturated fatty acid, docosahexenoic acid, methyl arachidonic acid,  $\alpha$ -linolenic acid, terpenoid, glycosides steroid, alkaloid, flavonoid, lectin, sterol and saponin (Shiomi et al., 1990; Westendorf, 1999; Teruya et al., 2001; Bhakuni & Rawat, 2005; Kelly, 2005; Lou et al., 2011; Sima & Vetvicka, 2011). Some authors predicted that the compounds have biological activities consist of antifungal, antibacterial, cytotoxicity, anticoagulant and immunostimulatory which are valuable for pharmacological industry (Andersson et al., 1989; Koyama et al., 1999; Faulkner, 2002; Kamel et al., 2005; Moore, 2006). Unfortunately, there was no empirical evidence reported that *A. planici* has immunostimulatory activities.

Research in immunostimulatory effect of the star fish (*A. planici*) on lymphocyte proliferation is very significant providing infectious disease has becoming one of worldwide health issues. The current research is expected to provide information about the prospective benefits of star fish *A. planici* as an immunostimulator. To be brief, the aims of this study was to identify the compounds from *A. planici* which has immunostimulatory effect on lymphocyte proliferation.

## 2. Material and Methods

### 2.1. Material

The samples were collected from Ternate Island at 3-10 m depth by scuba diving. The specimen was identified at Animal Taxonomy Laboratory, Faculty of Biology, immunostimulatory test at Integrated Research Testing Laboratory and identification of chemical compound at Organic Chemical Laboratory at Faculty of Mathematics and Natural Sciences University of Gadjah Mada Yogyakarta. Materials for this research were water, methanol (p.a, JT Baker), n-hexane (p.a, JT Baker), ethyl acetate (p.a, JT Baker), chloroform (p.a, JT Baker), silica gel (60-200 mesh, JT Baker), TLC (60 F-254, E. Merck), 96 well microplate, LC 30 mice balbc weigh 20 gram. Medium for cultivation immunomodulatory test were RPMI-1640 (Sigma), PHA-HA16 (Murex), penicillin streptomycin (Gibco) 2%, MTT-5655 (Sigma), 0.04 M isopropanol, 2 ml chloride ammonium, 2 ml/g bicarbonate sodium.

## 2.2. Methods

### 2.2.1. Isolation and identification

#### a. Extraction and partition

*Acanthaster planici* (700 g) was cut into small pieces wherein 300 gram was extracted by maceration method in methanol 90% and 400 g was extracted with water (24 hours) at room temperature. The two types of solvent were used for the reason that methanol and water could draw out the polar compounds. Afterwards, the extracts were evaporated in rotary evaporator (35 °C). These extracts were freeze dried and the rendement was then calculated. Result showed that 65 g of methanol extract had 21.7% rendement, while 40 g of water extract had 10% rendement.

Furthermore, the activities of methanol extract and water extract was tested. The test result showed that methanol extract had higher activity than that of water extract. Methanol extract was then partitioned with 187.5 ml hexane and 375 ml methanol to obtain hexane fraction. Subsequently, the 375 ml methanol was also partitioned with 187.5 ml chloroform and 187.5 ml water to obtain chloroform fraction and water fraction. At the end, the 3 fractions (hexane, chloroform and water) were evaporated and freeze dried to calculate the rendements. The result yielded that 13 gram of hexane fraction has 4.3% rendement, 22 of gram chloroform fraction has 7.3% rendement, while 15 gram of water fraction has 15%. Furthermore, the activity of each fraction was also tested.

#### b. Fractionation

As having the highest activity, hexane fraction was chromatographed on glass column (50 cm) packed with silica gel 60 (0.2-0.5 mm). Elution was carried out using gradient 75% hexane to 25% ethyl acetate. Fractions were collected and checked by thin layer chromatography (TLC) by mobile phase hexane and ethyl acetate (7:3 v/v). The fractions with the same TLC spot were then combined which resulted in 3 fractions. The result of evaporation and drying of the 3 fractions yielded that 2.1 gram of fraction 1 has 0.7% rendement, 2 g of fraction 2 has 0.67% rendement and 1.7 g of fraction 3 has 0.57% rendement. Subsequently, fraction 1, 2 and 3 were subjected for further bioassay.

#### c. Gas Chromatography-Mass Spectrometry (GC-MS)

Fraction with the highest activity was chosen as active fraction. The active fraction was then analyzed

by GC-MS-QP2010S Shimadzu equipped with RTX-5 MS column with 30 m length and 0.22 mm of internal diameter. The carrier gas used in this instrument was helium. The conditions of GC-MS instruments that used were 3200 °C temperature injector, 13.7 kPa pressure, 40 ml/minute total flow, 0.50 ml/minute column flow, 25.90 cm/second linear speed, 3 ml/minute purge flow, 73.0 split ratio, programmed column temperature from 700 °C (hold for 5 minutes) until 3000 °C (hold for 52 minutes), with the rate of temperature increase reached 100 °C/minute, the temperature of ion source reached 2500 °C and interface temperature reached 3200 °C.

### 2.2.2. Immunostimulatory activity test

Medium used to culture lymphocytic cells was 1 sachet RPMI 1640, 2 ml/g bicarbonate sodium and 2 ml/g herpes. Water was added to this mixture until reached 1000 ml water and was screened by microfilter (0.45 µm). Before used, the basic media was also mixed with 5 % serum, which subsequently called as complete media.

The culture lymphocytic cells were obtained from the spleen organ of 2 month old mice (20 g) from the Integrated Research and Testing Laboratory UGM. The mice were killed by perversical dislocation using chloroform and the abdominal was opened to take the spleen. The spleen blood was taken by injecting RPMI inside the spleen, lymphocyte liquid and RPMI were suspended and subsequently centrifuged at 1200 rpm for 10 minutes.

Lymphocytic cells that precipitated were diluted by 10 ml complete medium solution. Pellet was suspended by 2 ml chloride ammonium to lyses the erythrocyte. Lymphocytic cells were calculated with hemacytometer and the viability was determined by trypan blue. Accordingly, the cell suspensions were obtained by 5 x 10<sup>5</sup>/ml density and were cultured on 96 well microplate by 200 µl/well.

Sample concentration of *A. Planci* used were 640 µl/ml, 320 µl/ml, 160 µl/ml, 80 µl/ml, 40 µl/ml and 20 µl/ml. Phytohaemagglutinin (PHA) used was as much as 5 µl/ml -10 µl/well and incubated for 48 hours and 72 hours in 5% CO<sub>2</sub> incubator at 37°C. The reaction was stopped by adding 0.04 M isopropanol (100 µl/well). The result was read by *ELISA reader* (350 nm). Absorbency or optical density (OD) of each sample was calculated based on % stimulation index. The formula was:

$$\% \text{ Stimulation Index (SI)} = \frac{\text{Sample absorbency} \times 100}{\text{Control absorbency}}$$

## 3. Result and Discussion

### 3.1. Immunostimulatory Activity

#### a. Crude extract and partition

The lymphocytic proliferation activity test was conducted using *in vitro* method, by counting the number of lymphocytic cells on the negative control which consist of lymphocyte cell culture without treatment, and positive control which contained PHA mithogen. Subsequently, comparing with the number of lymphocytic cells in the media with sample, therefore, lymphocytic proliferation activity in the sample could be observed by calculating its percentage of stimulation index (SI) (Tizard, 2000; Wiedosari, 2005; Aminin et al., 2006).

The result of lymphocyte proliferation of methanol extract showed that at 48 hours and 72 hours by 73% (80 µl/ml) and 69.4% (320 µl/ml) increase. Meanwhile, of water extract at 48 hours and 72 hours by 73.5% (320 µl/ml) and 65.3% (320 µl/ml) increase. The increasing of lymphocyte proliferation at 48 hours and 72 hours of methanol extract and of water extract were

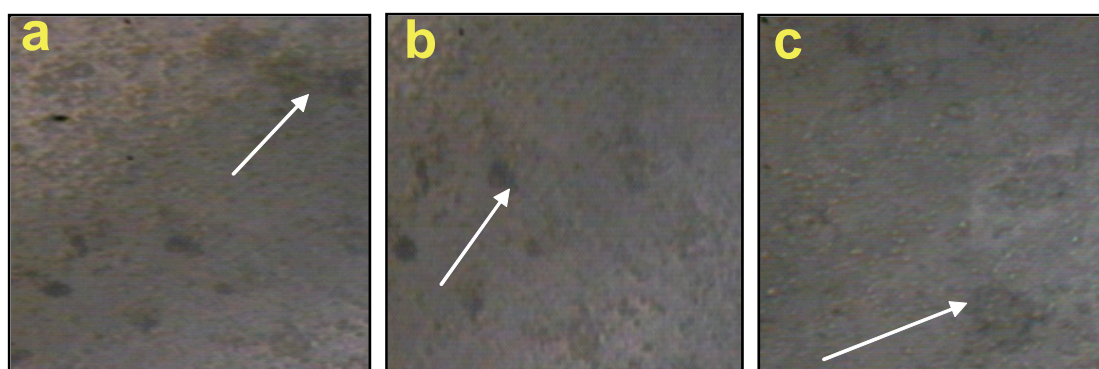


Figure 1. Lymphocyte proliferation of a. Methanol extract (640 µl/ml), b. Water extract (640 µl/ml), c. PHA (160 µl/ml).

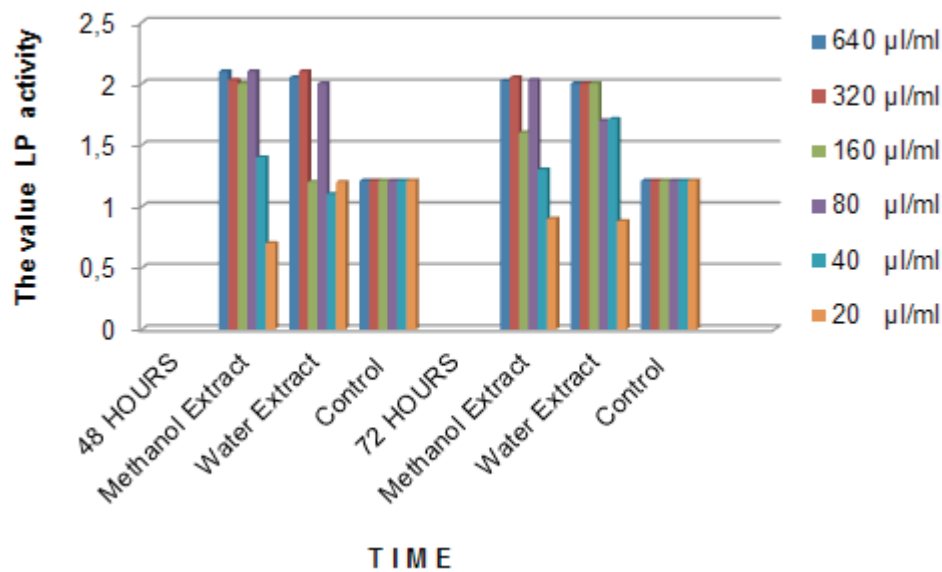


Figure 2. Lymphocyte proliferation activity at 48 hours and 72 hours from methanol extract, water extract and control.

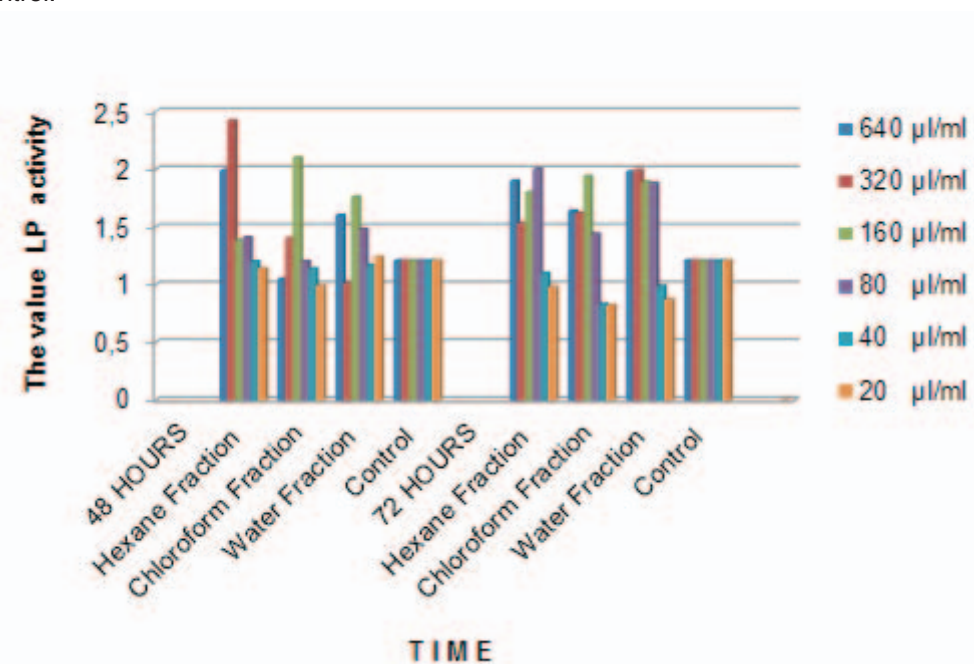


Figure 3. Lymphocyte proliferation activity at 48 hours and 72 hours of chloroform fraction, hexane fraction, water fraction and control.

higher than that of control. The lymphocyte proliferation activity 48 hours and 72 hours from methanol and water extracts could be seen in Figure 2.

Partition of methanol extract yielded 3 fractions (hexane, chloroform, and water). Hexane fraction was able to increase the lymphocyte proliferation at 48 hours and 72 hours by 100% (640 µl/ml) increase and 65% (80 µl/ml) increase compared to control. Chloroform fraction was able to increase the

lymphocyte proliferation at 48 hours and 72 hours by 71% (160 µl/ml) increase and 60.3% (160 µl/ml) increase compared to control. Meanwhile, water fraction was able to increase lymphocyte proliferation at 48 hours and 72 hours by 45% (160 µl/ml) increase and 64% (320 µl/ml) increase compared to control. The lymphocyte proliferation activity at 48 hours and 72 hours of chloroform fraction, hexane fraction and water fractions could be seen in Figure 3.

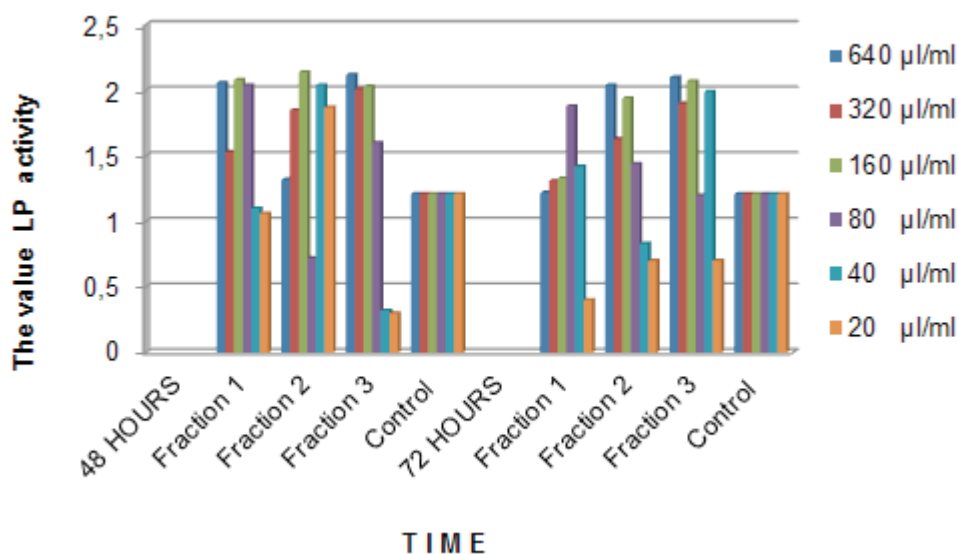


Figure 4. Lymphocyte proliferation activity at 48 hours and 72 hours from fraction 1, fraction 2, fraction 3 and control

### b. Fractionation

Fractionation of hexane fraction resulted 3 fractions. Fraction 1 was able to increase lymphocyte proliferation at 48 hours and 72 hours by 71.9% (160 µl/ml) and 55% (80 µl/ml) increase. Fraction 2 was able to increase lymphocyte proliferation at 48 hours and 72 hours by 76.8% (160 µl/ml) and 68.5% (640 µl/ml) increase. Meanwhile, fraction 3 was able to increase lymphocyte proliferation at 48 hours and 72 hours by 75% (640 µl/ml) and 73% (640 µl/ml) increase. The increasing of lymphocyte proliferation at 48 hours and 72 hours of 3 fractions were higher than that of control. The result showed that fraction 2 had the highest activity compared to fraction 1 and fraction 3. The lymphocyte proliferation activity at 48 hours and 72 hours of 3 fractions could be seen in Figure 4.

### 3.2. Identification of Chemical Compound from Active Fraction

The bioactive compounds of fraction 2 as the active fraction, were analyzed by GC-MS. The dominant peaks at different retention times that analyzed and identified from fraction 2 were methyl arachidonat acid, eicosapentaenoic acid and 9-docosahexanoic acid. Fatty acid such as methyl arachidonat acid, eicosapentaenoic acid and docosahexanoic acid had been used as proliferation of T cells inhibition, Interleukin 5 and 13 Inhibition and Macrophage phagocytosis inhibition (Pablo & Alvarez, 2000; Chapkin et al., 2014).

According to Schott and Huang (2012), the omega-3 (n-3) fatty acids docosahexaenoic acid and eicosapentaenoic acid, along with  $\alpha$ -linolenic acid and antioxidants modulated systemic inflammatory response and improved oxygenation and outcomes in patients with acute lung injury.

Luo et al. (2011) reported that *A. planici* had low lipid content, it had a good profile of fatty acid composition, which was manifested by the results that unsaturated fatty acids reached at 59.84 to 68.36% of total fatty acid and polyunsaturated fatty acids accounted for half of the unsaturated fatty acids. Also, polyunsaturated fatty acids contain timnodonic acid (EPA) (C20:5  $\omega$ -6, 1.31 to 2.73%) docosahexaenoic acid (DHA) (C22:6  $\omega$ -3, 0.89-1.71%) which have important physiological functions to humans and animals. Fatty acid compounds in *A. planici* derived from algae and coral became major food of these organisms (Teruya et al., 2001).

In the current study, chromatogram, peak MS and chemical constituents identified from the active fraction could be seen in Figure 5-11.

### 4. Conclusion

The study concluded that starfish *A. planici* from Ternate Island is a potential source of immunostimulator. Results showed that the highest activity of crude extract was methanol extract and the highest activity of 3 partition fractions was hexane fraction. Meanwhile, the fractionation of hexane fraction

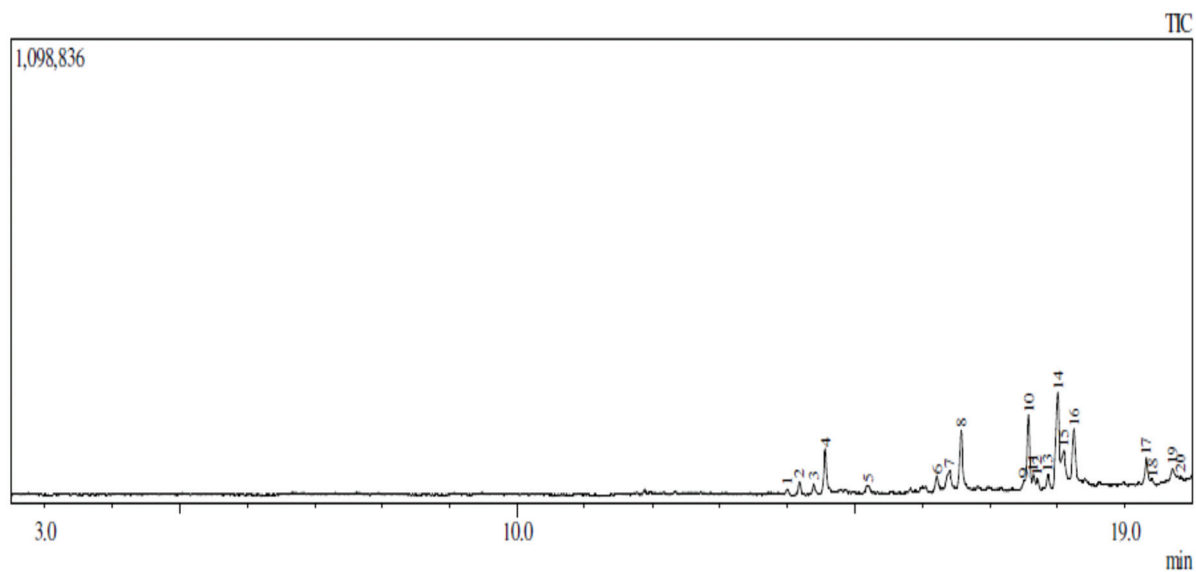


Figure 5. Chromatogram active fraction of *A. planci*.

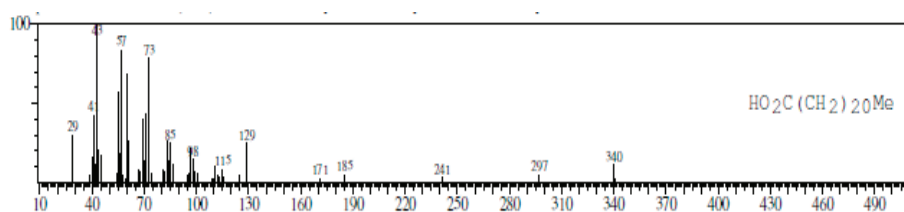


Figure 6. Peak MS no:8 retention time 14.542 minute.

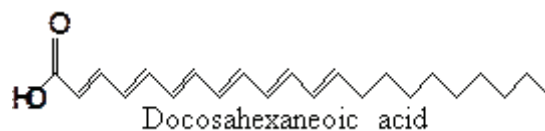


Figure 7. Prediction chemical constituents structure peak no: 8 (MW. 340).

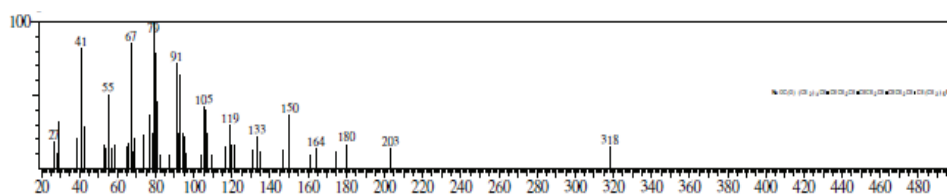


Figure 8. Peak MS no:10 retention time 18.100 minute.

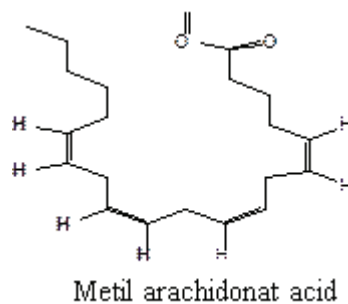


Figure 9. Prediction chemical constituents structure peak no:10 (MW.318).

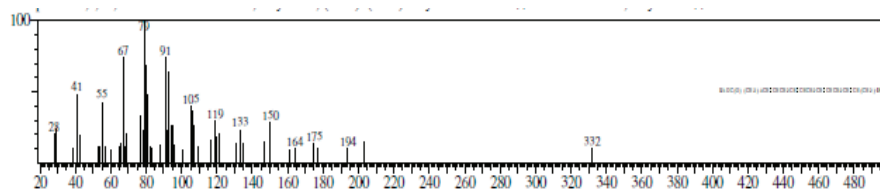


Figure 10. Peak MS no: 14 retention time 18.000 minute.

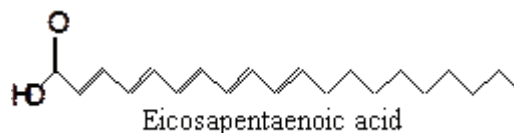


Figure 11. Prediction chemical constituents structure peak no:14 (MW.332).

yielded 3 fractions in which fraction 2 had the highest activity. At all observation stages, the highest activities were stronger at 48 hours than that of 72 hours. As a final point, the results that analyzed and identified by GC-MS of active fractions were contained methyl arachidonat acid, eicosapentaenoic acid and 9-docosahexanoic acid.

## References

- Aminin, D. L., Pinegin, B. V., Pichugina, L. V., Zaporozhets, T. S., Agafonova, I. G., Boguslavski, V. M., Silchenko, A. S., Avilov, S. A., & Stonik, V. A. (2006). Immunomodulatory properties of Cumaside. *International Journal Immunopharmacology*, 6, 1070-1082.
- Andersson, I., Bohlin, L., Lorizzi, M., Raffaele, R., Minale, L., & Moreno, L. W. (1989). Biological Activity of Saponins and Saponin-like Compounds from Starfish and Brittle-Stars. *Journal of Toxicology*, 27 (2), 179-188.
- Bakhuni, D. S., & Rawat, D. S. (2005). *Bioactive Marine Natural Product* (pp. 48-51), Anamaya Publishers, New Delhi, India
- Chapkin, R. S., Laurie, A. D., Lan, L., Brad, R., Joanne L., & David, N. M. (2014). Immunomodulatory Effects of (n-3) Fatty Acids: Putative Link to Inflammation and Colon Cancer 1-3. *Journal of Nutrition*, 7(12), 0022-3166.
- Faulkner, D. J. (2002). Marine Natural Products. *Journal of Natural Products*, 17(5), 17-55.
- Fraser, N., Crawford, B., & Kusen, J. (2000). *Best Practices Guide For Crown-of-Thorns Clean-Ups* (pp 5-38). University of Rhode Island, Narragansett, Rhode Island, USA.
- Houk, P., Bograd, S., & Woesik, R. V. (2007). The Transition zone chlorophyll frontean Trigger *Acanthaster planci* Outbreaks in the Pacific Ocean: Historical confirmation. *Journal of Oceanography*, 63, 149-154.
- Kamel, H. N. & Slattery, M. (2005). Terpenoids of *Sinularia*: Chemistry and Biomedical Applications. *Journal of Pharmaceutical Biology*, 43, 253-269.
- Kelly, M. S. (2005). Echinoderms: *Their Culture and Bioactive Compounds*. In Matranga, V., Muller (Editors). *Echinoderms: Marine Molecular Biotechnology* (pp 139-153). Berlin Heidelberg, Germany: Springer.
- Koyama, T., Katsuhiko, N., Yoko, A., & Matao, S. (1999). Analysis for Sites of Anticoagulant Action of Plancinin, a New Anticoagulant Peptide Isolated from the Starfish *Acanthaster planci*, in the Blood Coagulation Cascade. *Journal of Pharmacology*, 903-215.
- Luo, P., Chao-qun, H. U., Jian-jun Xia, Chun-hua, R., & Xiao-Jiang. (2011). Chemical Constituent Analysis of the Crown-of-Thorns Starfish *Acanthaster planci* and Potential Utilization Value of the starfish as feed ingredient for Animal. *African Journal of Biotechnology*, 10(62), 13610-13616.
- Mendonca, Al. J., Al Ajmi, Al Muharrami., Al Areimi, & Al Aghbari. (2010). Persisten and Expanding Population Outbreaks of the Corallivorous Starfish *A. Planci* in the Northwestern Indian Ocean. *Journal of Environment Zoological*, 49(1), 108-123.
- Moore, S. (2006). Biosynthesis of Marine Natural Products Macroorganisms (Part B). *Journal The Royal Society of Chemistry*, 23(2), 615-629.
- Pablo, M. A. & Álvarez, G. D. (2000). Modulatory effects of dietary lipids on immune system functions. *Journal of Immunology and Cell Biology*, 78, 31-39.
- Sapp, J. (1999). *What is Natural. Coral Reef Crisis* (pp. 66-76). Oxford University Press. New York, USA.
- Schott, C. K. & Huang, D. T. (2012). w-3 fatty acids, a-linolenic acid, and antioxidants: immunomodulators or inert dietary supplements. *Journal Club Critique*, 16(6), 325.
- Shiomi, K., Yamamoto, K., Yamanaka, H., Kikuchi., & Kenjiro, K. (1990). Liver Damage by the Crown-of-Thorns Starfish (*Acanthaster planci*) Lethal Factor. *Journal of Toxicology*, 28(5), 469-475.
- Sima, P., & Vetvicka, V. (2011). Bioactive substances with anti-neoplastic efficacy from marine invertebrates:

- Bryozoa, Mollusca, Echinodermata and Urochordata. *World Journal of Clinical Oncology*, 11, 362-366.
- Teruya, T., Kiyotake, S., Tomoyuki, K., Yoshikatsu., & Daisuke, U. (2001). Arachidonic acid and a-linolenic acid, Feeding Attractants for the Crown-of-Thorns Sea Star *Acanthaster planci*, from the Sea Urchin *Toxopneustes pileolus*. *Journal of Experimental Marine Biology and Ecology*, 266, 123-134.
- Tizard, I. R. (2000). *Immunology: An Introduction* (6th Edition). New York: Saunders College Publishing.
- Westendorf, J. (1999). Natural Compounds. *Journal Toxicology*, 43(7), 234-235.
- Wiedosari, E. (2005). *Difference of expression cytokine mRNA between sheep and merine on fasciola infection gigantic*. Dissertation. Doctoral programme, Bogor Agricultural University. Bogor, Indonesia .