PHARMACIA

Pharmacia 71: 1–9
DOI 10.3897/pharmacia.71.e113504

Research Article

Action of Ergost-7-en-3- ol from (*Acanthaster planci*) stimulation of activity peritoneal macrophages

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Received 30 September 2023 ♦ **Accepted** 2 February 2024 ♦ **Published** 25 April 2024

Citation: Achmad MJ, Samman A, Akbar N (2024) Action of Ergost-7-en-3- ol from (*Acanthaster planci*) stimulation of activity peritoneal macrophages. Pharmacia 71: 1–9. https://doi.org/10.3897/pharmacia.71.e113504

Abstract

An is a compound capable of influencing the human immune system because it can maintain and restore the balance of the immune system by stimulating the immune system through phagocytosis, the complement system, IgA antibody secretion, the release of interferon α and γ, T and B lymphocytes, specific antibody systems, and cytokines. Immunomodulators can be obtained from natural sources, one of which is *Acanthaster planci*, which is one of the marine organisms that has important bioactive substances that can act as immunomodulators. The purpose of this research is to determine the immunomodulatory activity of chloroform fractions FrKl3.1.3.1 and FrKl3.1.3.2 and to identify the immunomodulatory compounds from the chloroform fraction FrKl3.1.3.1 of *A. planci*. The results of the study showed that the macrophage test of the FrKl3.1.3.1 fraction had higher activity compared to FrKl3.1.3.2, while the spectroscopic data analysis results showed that UV-VIS had a maximum absorption at a wavelength (λmax) of 240 nm, FTIR data (cm-1) showed the presence of hydroxyl (-OH) groups, alkene groups (double bonds (C=C)), and aliphatic C-H groups. The 1H-NMR data (CDCl3, 500 MHz) showed six methane groups, ten aliphatic methylene groups (-CH2-), nine geminal methylene groups, one methylene group with a double bond, and 13C-NMR and DEPT 135 data (CDCl3, 125 MHz) indicated that the *A. planci* FrKl3.1.3.1 fraction had 28 carbon atoms consisting of six methyl (-CH3) carbon atoms, ten methylene (-CH2) carbon atoms, and nine methine (-CH-) carbon atoms. From the spectroscopic analysis data, it is known that the chloroform fraction of *A. planci* FrKl3.1.3.1 is *ergost-7-en-3-ol* compound.

Keywords

Star fish, *A. planci*, immunomodulator, macrophages, steroid, ergost

Introduction

The immune system in the human body is continually exposed to infectious agents that invade and lead to diseases. This immune system serves to safeguard the body and aid in the recovery and repair of damaged cells when infections occur (Roit et al. 1993; Abbas et al. 2000; Achmad et al. 2018).

In a normal immune system, the body maintains a balance among its components, so when there's an infection, it doesn't result in permanent damage because the immune system can control and eliminate the infecting agents (Corwin 2009). To maintain this balance, the body requires substances from natural compounds or immunomodulators that can modify the immune response by activating both natural and adaptive defense mechanisms.

This includes restoring any disrupted immune system imbalance caused by infections through processes such as stimulating the immune system, phagocytosis, the complement system, IgA antibody secretion, the release of interferon α and γ, T and B lymphocytes, specific antibody systems, and cytokines (Roit et al. 1993; De Pablo et al. 2000; Tringal 2001; Petrunov et al. 2007; Ghelani et al. 2022).

Due to their unique and essential secondary metabolic components, including triterpenes, sterols, saponins, glycosides, alkaloids or flavonoids, natural products from marine organisms could serve as immunomodulators (Shiomi et al. 1985; Shiomi et al. 1990; Tringali 2001; Faulkner 2002; Kamel et al. 2005; Kelly 2005; Moore 2006; Achmad et al. 2018).

Acanthaster planci, an *echinoderm* phylum member, is a possible immunomodulator derived from marine organisms. *A. planci* is an alga that forms part of coral reef ecosystems and its presence is essential to maintain a balanced number of corals in the ecosystem. However, the abundance of *A. planci* on coral reefs can present a serious threat to their survival (Houk et al. 2007; Nugues and Bak 2009; Mendonca et al. 2010; Tokashi et al. 2011). This organism has the potential to act as an immunomodulator due to its unique primary metabolite compound content (Bhakuni and Rawat 2005; Yokota 2005; Mayer et al. 2009; Watanabe et al. 2009; Achmad et al. 2014; Achmad et al. 2018; Achmad et al. 2023). This study aims at determining the immunomodulatoric activity of *A. planci*'s chloroform fractions FrKl3.1.3.1 and FrKl3.1.3.2, as well as finding its immunomodulating compounds.

Material and methods

The *A. plan*ci samples were collected from the waters of Ternate Island, North Maluku, Indonesia, at depths ranging from 5 to 10 meters using scuba diving in August 2021 (Fig. 1). The samples were assigned location-specific codes and were stored under cold conditions (-10 °C). Identification was carried out at the Animal and Plant Taxonomy Laboratory, Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia, with specimen code no. AP-01. Extraction was performed using the maceration method (12 hours, repeated three times) with methanol, hexane, and chloroform solvents at a ratio of 1:2 (w/v), following the procedures outlined by Achmad et al. in 2014 and Nursid et al. *in* 2021. Sample identification was conducted based on the *echinoderm* identification key provided by Frazer et al. in 2000.

The test for macrophage phagocytosis

By the method described by Aminin et al. (2010), and Achmad et al. (2014), a macrophage activity test has been performed. At a dose of 0.4 mg per kg bodyweight of mice, a chloroform fraction of *A. planci* has been administered. The mice were killed on the 4th day after injection of *A. planci* compound. They placed mice in a supine position and filled their peritoneal compartments with 70% alcohol. After that, 10 ml RPMI medium was injected

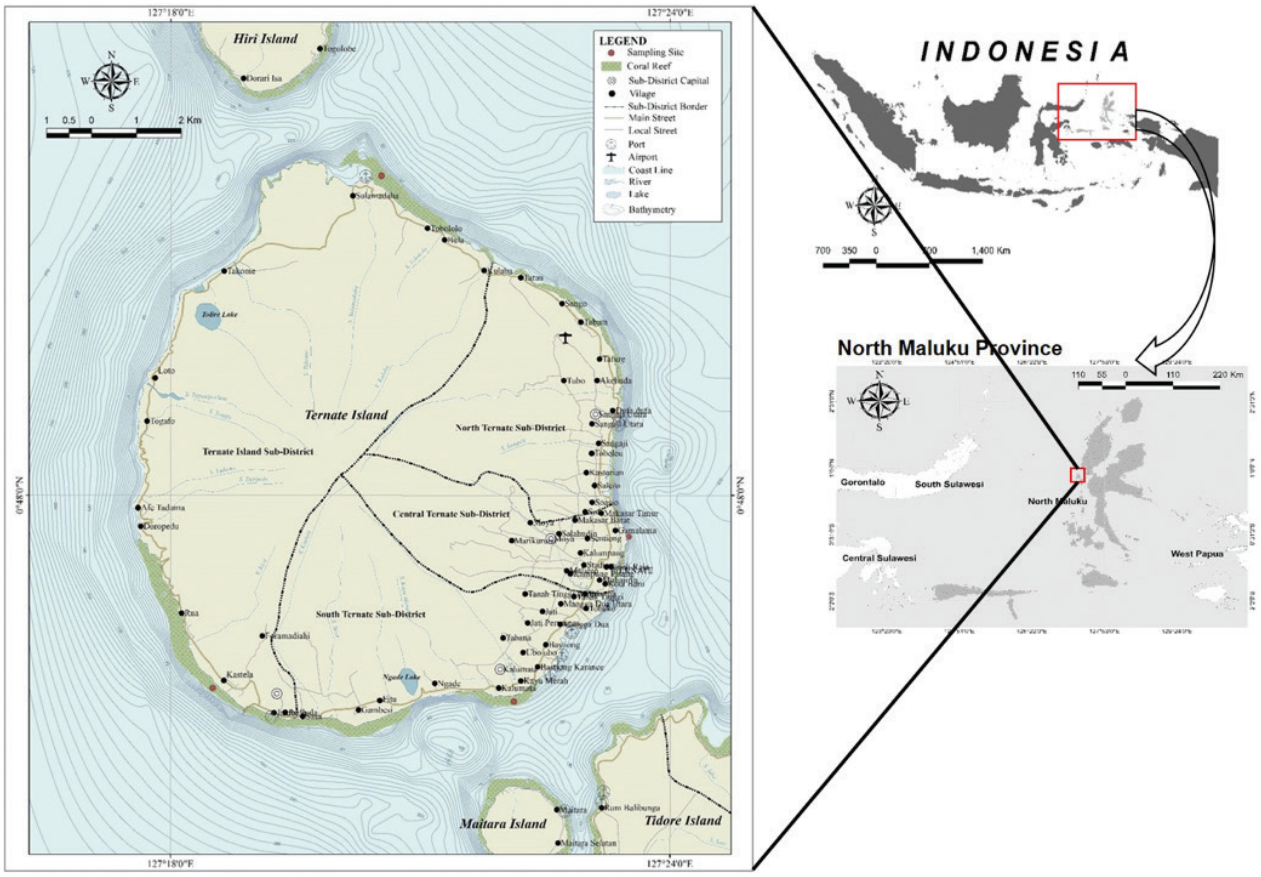

Figure 1. A map of the sampling locations

into the abdominal region and a 15ml syringe with gentle pressure on the abdomen was used to collect fluid from it. In a 15ml falcon tube, the peritoneal fluid was taken and centrifuged at 1200 rpm for 10 minutes. The pellet formed was resuspended in 3 ml of culture medium (FBS 10%, Penstrep 2%, Fungison 0.5%, and RPMI solvent). After that, a hemocytometer was used to determine the number of cells and the viability of the cells was assessed using trypan blue.

The macrophage cell culture medium, 50 µl, and RPMI, 950 µl, were introduced into a microplate with 24 wells, each of which had been equipped with a coverslip. This assembly was incubated for 24 hours. Subsequently, the cells were rinsed with RPMI, and a suspension of latex particles (20 µl) was added to each well. This was followed by a 60-minute incubation period in a CO_2 incubator set at 5% CO_2 and 37 °C. The macrophage cells formed in each well were then washed three times with PBS to remove non-phagocytosed latex particles. The cells were air-dried at room temperature and fixed with absolute methanol for 30 seconds. Afterward, the methanol was discarded, and the coverslips were allowed to air dry. Giemsa staining solution (20% Giemsa, 1 ml) was added to each well and incubated for 20 minutes. Following this, the coverslips were rinsed with distilled water and allowed to dry at room temperature. The dried coverslips were observed under a microscope at 400× magnification to count 100 macrophage cells that had phagocytosed latex particles. The Phagocytic Index (PI) and Phagocytic Capacity (PC) were then determined using formulas adapted from Aminin et al. 2006; Jensch-Junior et al. 2006; Anwei et al. 2008; Aminin et al. 2010; Achmad et al. 2014; Achmad et al. 2018; Nursid et al. 2021.

$$\text{Phagocytic Capacity} = \frac{\text{The number of macrophages that have phagocytosed (engulfed) particles}}{\text{The total number of macrophages that were counted}} \times 100$$

$$\text{Phagocytic Index} = \frac{\text{The number of latex particles that were phagocytosed by macrophages}}{\text{The number of macrophages that have undergone phagocytosis}} \times 100$$

The measurement of a Liquid Chromatography Mass Spectrometry (LC-MS)

The mass spectra measurement of the chloroform fraction FrKl3.1.3.1, at a quantity of 0.2 mg, was conducted using an LC-ESI-ToF-MS instrument with a Sunfire column measuring 4.6 × 150 mm. The analysis was carried out under isocratic conditions with a mobile phase consisting of H2O + formic acid: acetonitrile = 45/55 v/v, at a flow rate of 0.7 ml/minute, and an injection volume of 10 µl.

The measurement of UV-VIS spectroscopy

Pure compounds are examined using a UV-visible spectrophotometer in the wavelength range (λ) of 200–400 nm. A quantity of 0.3 mg is dissolved in 3 ml of solvent. Then, 0.3 ml of this solution is taken and diluted to a total volume of 3 ml. The spectrum is recorded and printed. This procedure allows for the measurement of the compound's absorption or transmission of light within the specified wavelength range, which can provide valuable information about its electronic structure and properties.

The measurement of Fourier-Transform Infrared (FTIR) spectroscopy

A quantity of 0.2 mg of the chloroform fraction sample *A. planci* FrKl3.1.3.1 is ground together with 200 mg of potassium bromide (KBr) until they are thoroughly mixed. Afterward, this mixture is pressed into a thin disc. The resulting pellet is then subjected to infrared spectroscopic analysis to obtain its infrared spectrum. This procedure is a common method for preparing solid samples for Fourier-Transform Infrared (FTIR) spectroscopy. The resulting spectrum will provide information about the sample's molecular vibrations and functional groups, which can be useful for identifying its chemical composition and structure.

The measurement of proton nuclear magnetic resonance (^1H-NMR)

The chloroform fraction *A. planci* FrKl3.1.3.1, weighing 5 mg, was subjected to washing with hexane and acetone to remove any fatty acids present in the chloroform fraction. Then, it was dissolved in CDCl3. Subsequently, ^1H-NMR and ^13C-NMR spectra were recorded. This analysis was carried out at the LIPI Organic Chemistry Laboratory in Serpong, using a 500 MHz NMR spectrometer, with a chemical shift range of up to 15.50 ppm, using CDCl3 as the solvent. This procedure is a common method for analyzing the nuclear magnetic resonance spectra of organic compounds, providing valuable information about the structure and connectivity of atoms within the sample.

Results

The macrophage test results indicate that the chloroform fraction *A. planc*i FrKl3.1.3.1 has higher activity compared to the chloroform fraction FrKl3.1.3.2. With these results, further spectroscopic analysis is conducted on the FrKl3.1.3.1 fraction to determine the chemical structure present in this fraction. The KF and IF values for FrKl3.1.3.1 and FrKl3.1.3.2 (Figs 2, 3).

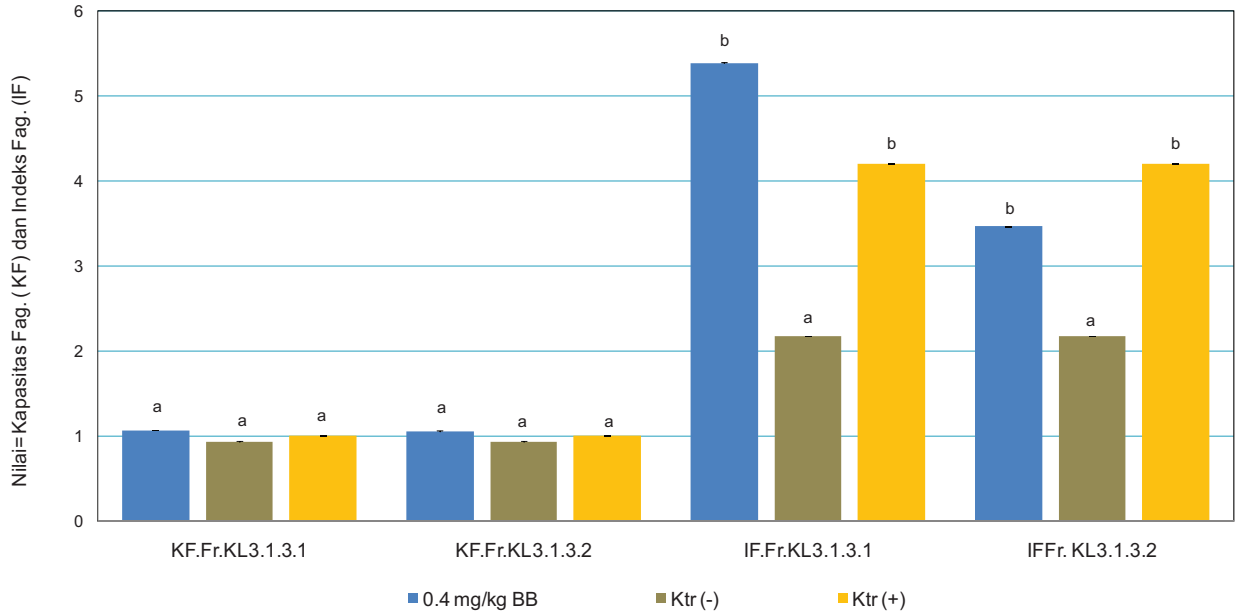

Figure 2. Value of phagocytic index (PI) and phagocytic capacity (PC) from FrKl3.1.3.1 and FrKl3.1.3.2.

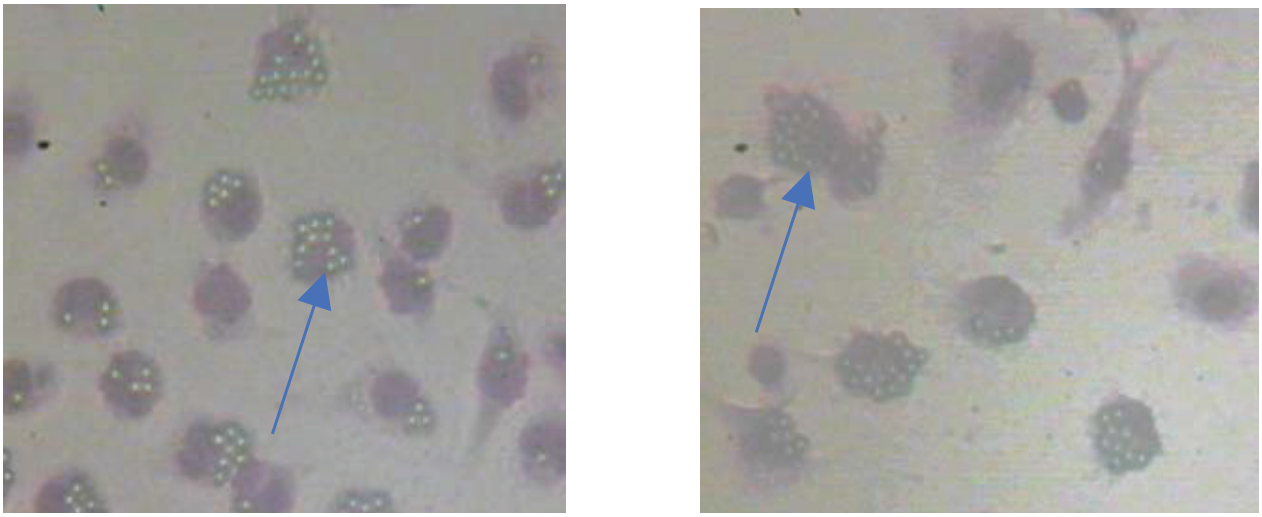

Figure 3. Macrophage activitas clorofom fraction *A. planci* FrKl3.1.3.1 and FrKl3.1.3.2.

The FrKl3.1.3.1 fraction, as the selected fraction, was further subjected to chemical compound identification using spectroscopic instrumentation to identify chemical functional groups and determine the chemical structure of the compound in FrKl3.1.3.1. The results from the LC-ESI-ToF-MS spectrum revealed a molecular ion peak at m/z 400.0389 [M-H] with a retention time of 5.725 (Fig. 4). The LC-MS chromatogram indicates that the compound FrKl3.1.3.1 exhibits four peaks, with the second peak being more dominant compared to the others (Fig. 5). Note: LCMS Shimadzu, kolom ODS 2.0 × 150 mm, gradien 10% ACN to 100% ACN (30'), 100% ACN (20'), flow rate: 0.2 ml/min, detector PDA

The UV-VIS spectrum of FrKl3.1.3.1 shows a maximum absorption peak at a wavelength (λmax) of 240 nm. The presence of a relatively small (weak) maximum absorption peak around λ 240 nm or higher is a characteristic feature of sterol compounds (Naewbanij et al. 1984). Based on the measured maximum wavelength (λmax) and referring to the literature on the maximum wavelengths of plant sterol compounds, it can be concluded that compound FrKl3.1.3.1 belongs to the sterol compound group (Fig. 6).

The compound FrKl3.1.3.1 has FTIR wavenumber (cm^-1) data, including 3298 cm^-1, indicating the presence of hydroxyl (-OH) groups, which is further supported by the C-O stretching peak at 1043 cm^-1. 3100–3200 cm^-1, suggesting the presence of alkene (double bond C=C) groups. 2870–2953 cm^-1, indicative of aliphatic C-H groups, supported by symmetric C-H bending vibrations at 1375 cm^-1 and asymmetric bending vibrations at 1448–1463 cm^-1 (Fig. 7).

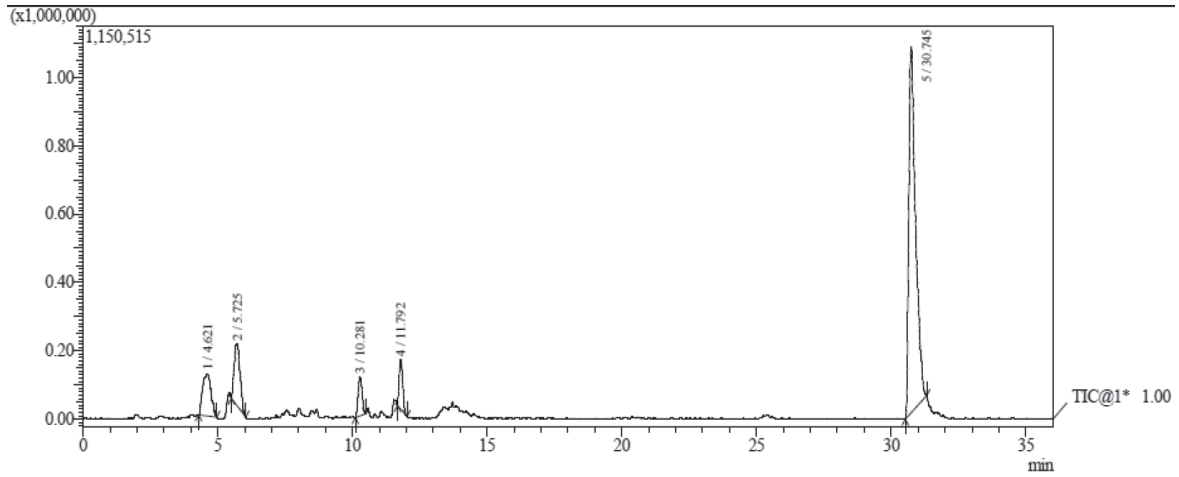

Figure 4. Cromatogram LC-MS FrKl3.1.3.1.

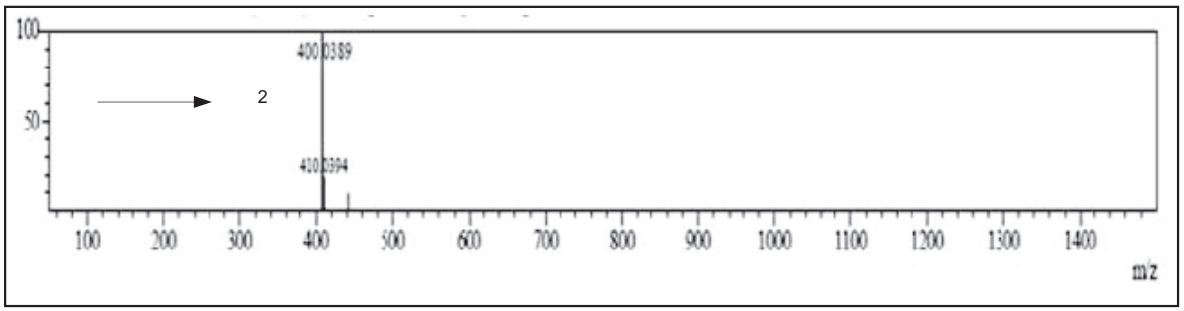

Figure 5. Peak MS no:2 retention time 5.725 minute (MW.400.0389) from FrKl3.1.3.1

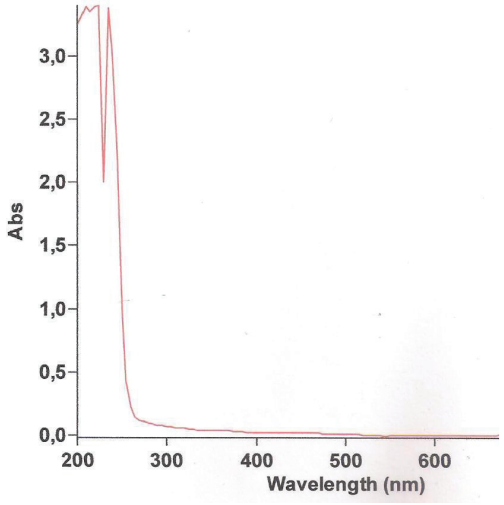

Figure 6. UV-Vis spectrum of FrKl3.1.3.1.

Based on the 1H-NMR data (CDCl3, 500 MHz) (Fig. 8), it is observed that the FrKl3.1.3.1 fraction exhibits a general sterol compound profile. The FrKl3.1.3.1 fraction has six methyl groups indicated by chemical shift peaks at δH 0.79 (3H, s); 0.53 (3H, s); 0.92 (3H, d); 0.85 (3H, d); 0.78 (3H, d); and 0.77 (3H, d) ppm. There are ten aliphatic methylene (-CH2-) groups, consisting of nine geminal methylene groups (two protons with different chemical shift values bound to the same carbon atom) in the range of δH 0.84–1.83 ppm and one methylene group with proton shifts at δH 2.01 (2H, dd, H-12) ppm. Additionally, there is one double bond (sp2) group at δH 5.17 (1H, dd, H-7), one -CH-O- group at δ 3.59 (1H, m, H-3), and seven methine (-CH-) groups at δH 1.38 (1H, m, H-5); 1.64 (1H, t, H-9); 1.78 (1H, dd, H-14); 1.23 (1H, m, H-17); 1.34 (1H, m, H-20); 1.22 (1H, m, H-24); and 0.84 (1H, m, H-25) (Table 1).

Based on the 13C-NMR and DEPT 135 (CDCl3, 125 MHz) data (Figs 8, 9) below, it is recognized that the FrKl3.1.3.1 fraction has 28 carbon atoms, consisting of six methyl carbon (-CH3) atoms, ten methylene carbon (-CH2) atoms, nine methine carbon (-CH-) atoms, including one oxygenated methine (HC-O) at δC 71.3 ppm, one sp2 methine at δC 117.6 ppm, and six sp3 methine carbon atoms at δC 40.5; 49.7; 55.2; 56.2; 36.8; 20.4, as well as three quaternary carbon atoms at δC 34.4; 43.6; and 139.8 ppm.

The correlation between protons and carbons, as well as correlations with neighboring carbon atoms within a distance of 3–4 bonds, can be observed based on two-dimensional correlations through HMQC and HMBC measurements (Fig. 8). Based on the information from UV, IR, LC-ESI-ToF-MS, 1H-NMR, 13C-NMR, DEPT, and HMQC spectra, the active compound is identified as a type of sterol compound called ergost-7-en-3-ol (Figs 10, 11).

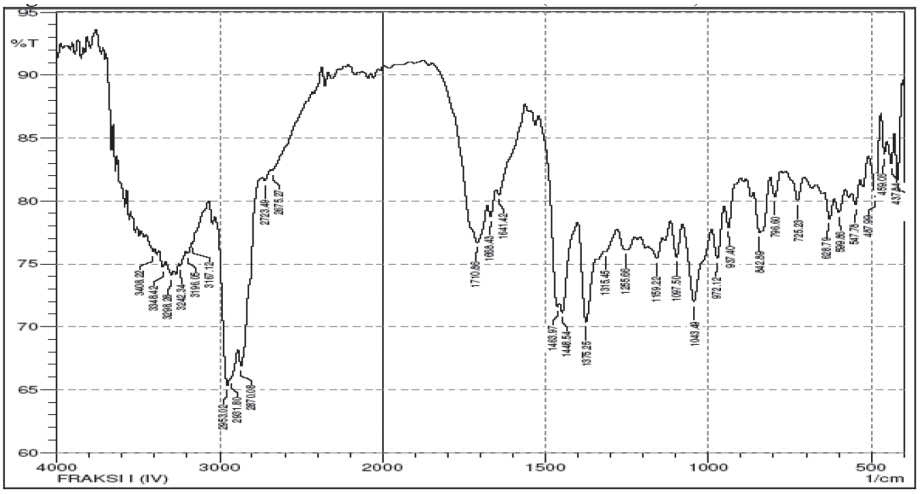

Figure 7. FTIR Spectrum of FrKl3.1.3.1.

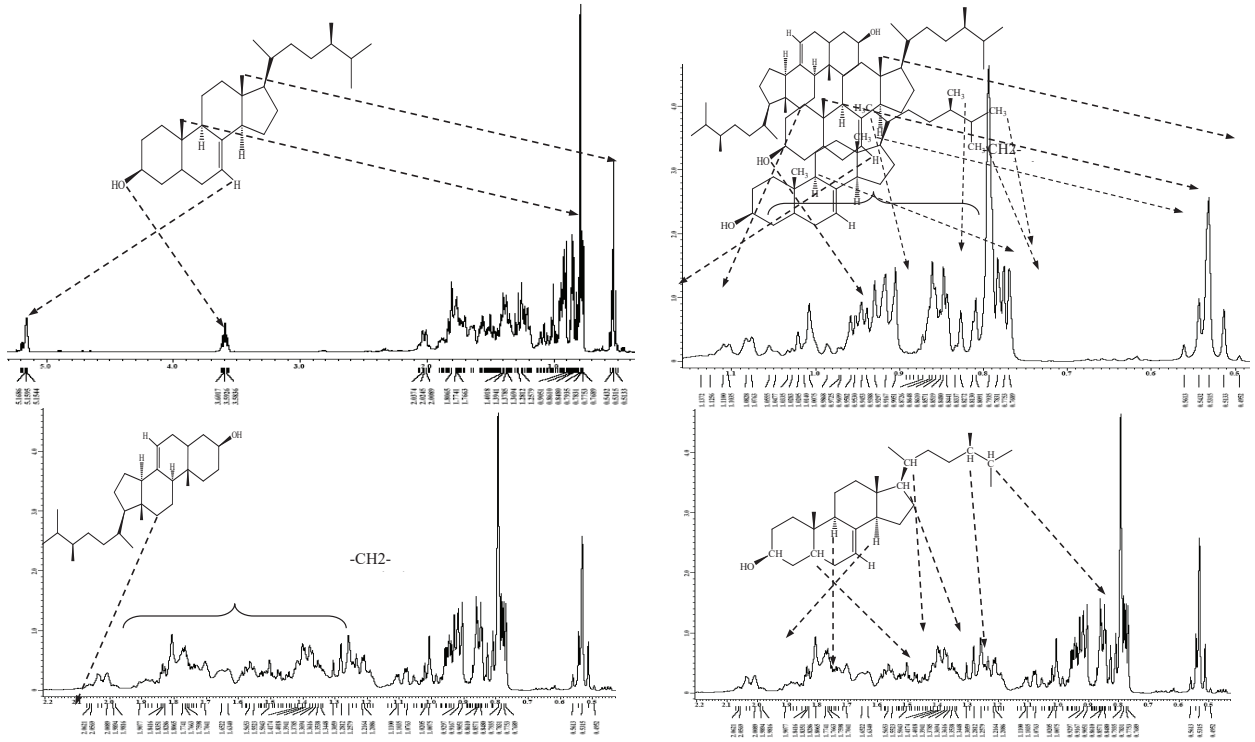

Figure 8. ^{1}H-NMR spectrum of FrKl3.1.3.1.

Discussion

The chloroform fraction of *A. planci* exhibits immunomodulatory activity based on the macrophage test. Compared to the positive control, this is evident by higher KF and IF values of the chloroform fraction. This indicates that marine organisms, especially echinoderms like sea cucumbers and *A. planci*, contain important secondary metabolites such as triterpenes, sterols, glycosides, and saponins (Bhakuni and Rawat 2005; Anwei et al. 2008; Aminin et al. 2008; Aminin et al. 2009; Watanabe et al. 2009; Aminin et al. 2010; Achmad et al. 2014; Achmad et al. 2018; Ghelani et al. 2022) with immunomodulatory properties.

Based on spectroscopic analysis, the name of the compound in the chloroform fraction from *A. planci* FrKl3.1.3.1 is ergost-7-en-3-ol, or IUPAC name (5,6-dimethylheptan-2-yl-10,13-dimethyl-2,3,4,5,6,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta(a)phenanthren-3-ol). This identification is further supported by the confirmation from GS-MS, which provided a molecular weight of 400.68012 g/mol and a molecular formula of C28H480. According to Sato et al. (1980), the sterol compound composition of *A. planci* includes similar relative retention times in Gas-Liquid Chromatography (GLC) as ergosta-5-en-3β-ol, 5α-ergost-7-en-3β-ol, 5α-ergosta-7,24 (28)-dien-3β-ol, and ergost-7-en-3-ol.

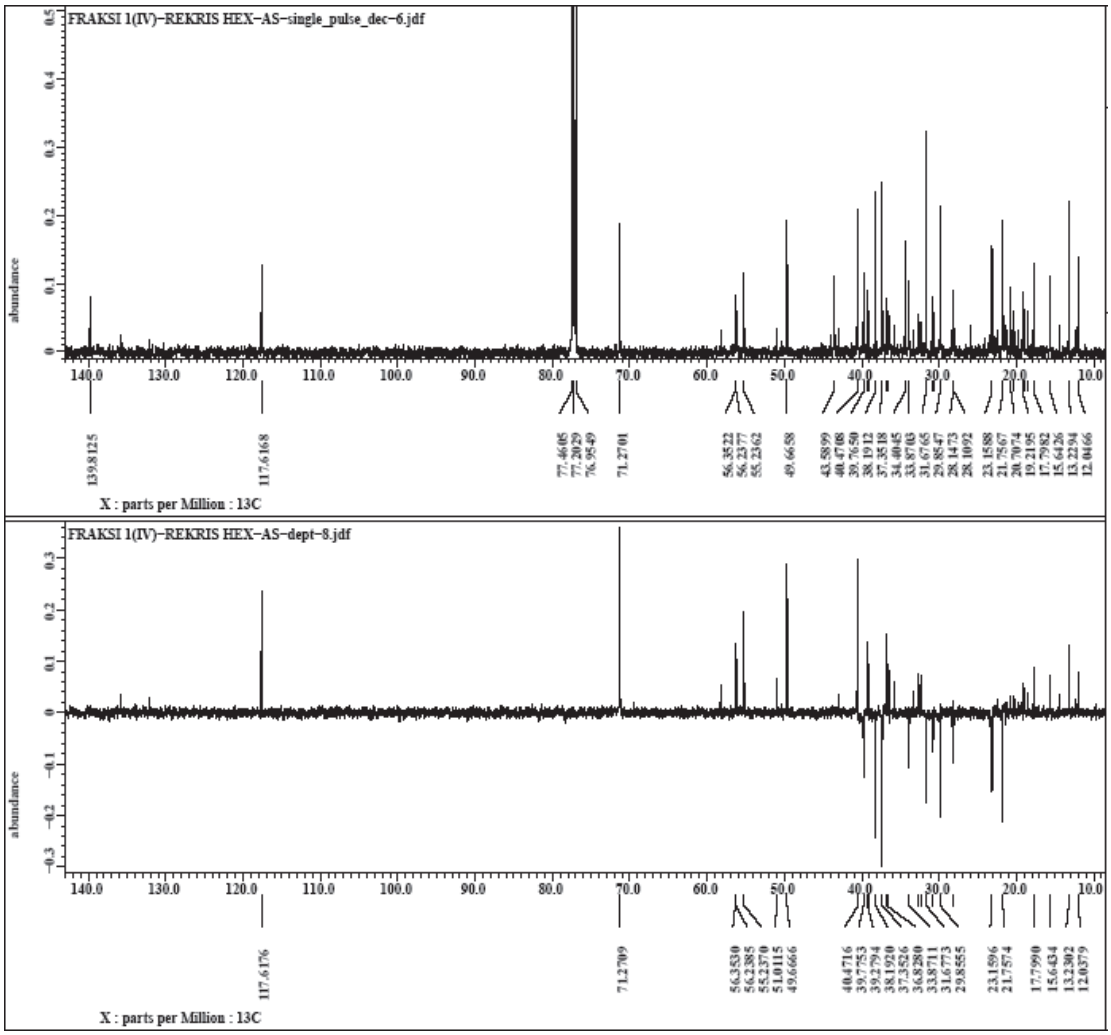

Figure 9. ^{13}C –NMR spectrum and DEPT 135 of Fr Kl3.1.3.1.

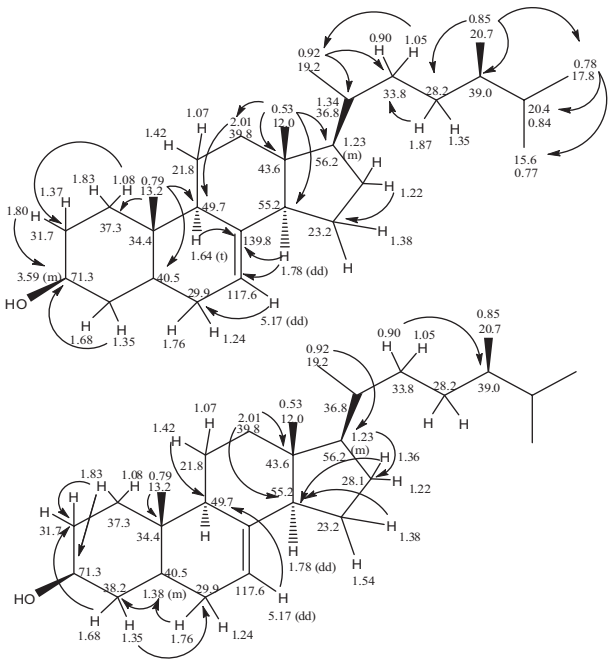

Figure 10. HMQC and HMBC correlation of ergost-7-en-3-ol compound.

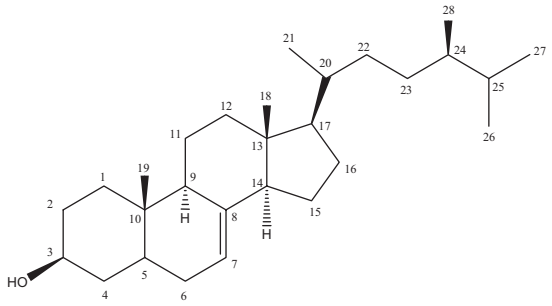

Figure 11. Chemical structure of ergost-7-en-3-ol compound.

Ergost-7-en-ol is a sterol compound that belongs to the triterpenoid group of terpenoids, characterized by a basic framework of a cyclopentane perhydrophenanthrene ring (Sato et al. 1980; Robinson 1995; Kanazawa 2001). According to Sato et al. (1980), sterol compounds from *A. planci* were first isolated by Sheikh and Djerassi in 1971, and up to 2012, 112 compounds have been discovered, with sterols dominating the composition (Fig. 10).

According to Teruya (2001), Kanazawa (2001), and Dewick (2004), sterol compounds in *A. planci* typically exhibit a highly complex mixture of C26, C27, C28, and

Table 1. Spectrum Data of ^{1H}-NMR and ^{13C}-NMR from Fr Kl3.1.3.1.

No.	Fr Kl3.1.3.1					
	δ_H (ppm) (ΣH, mult., *J* Hz)	δ_C (ppm)	HMBC			
			1	2	3	4
1	1.08 (1H, dt)	37.3	31.7			
	1.83 (1H, m)			71.3		
2	1.37 (1H, m)	31.7				
	1.80 (1H)		71.3			
3	3.59 (1H, m)	71.3				
4	1.35 (1H, m)	38.2	71.3			
	1.65 (1H, m)		31.7			
5	1.38 (1H, m)	40.5	38.2			
6	1.24 (1H)	29.9				
	1.76 (1H, m)		40.5			
7	5.17 (1H, dd)	117.6	29.9	49.7		
8	–	139.8				
9	1.64 (1H, dd)	49.7	139.8			
10	–	34.4				
11	1.07 (1H, dt)	21.8				
	1.42 (1H, dt)		49.7			
12	2.01 (2H, m)	39.8				
13	–	43.6				
14	1.78 (1H, m)	55.2	117.6	139.8		
15	1.38 (1H, m)	23.2	55.2			
	1.54 (1H, m)					
16	1.22 (1H, m)	28.1	55.2			
	1.36 (1H, m)		23.2			
17	1.23 (1H, m)	56.2	28.1			
18	0.53 (3H, s)	12.0	39.8	43.6	55.2	56.2
19	0.79 (3H, s)	13.2	37.3	40.5	49.7	
20	1.34 (1H, m)	36.8				
21	0.92 (3H, d)	19.2	33.8	36.8	56.2	
22	0.90 (1H)	33.8	39.0			
	1.05 (1H, dt)		19.2			
23	1.35 (1H, m)	28.2				
	1.87 (1H, m)		33.8			
24	1.22 (1H, m)	39.0				
25	0.84 (1H, m)	20.4				
26	0.77 (3H, d)	15.6				
27	0.78 (3H, d)	17.8	15.6	20.4		
28	0.85 (3H, d)	20.7	17.8	28.2	39.0	

Note: s= single, d=doblet, m= multi, dd=doblet-doblet, td = triple doblet.

C30 sterols. The dominant sterol compounds in these starfishes are sourced from the corals, which are their primary food, with ergosterol being one of the significant sterols derived from corals and algae that act as intermediaries in the sterol biosynthesis of *A. planci*.

In general, sterol biosynthesis in the class *asteroidea* (starfish) starts from the mevalonate pathway, which begins with acetic acid as the initial compound in the biosynthetic process. This pathway is activated by coenzymes and undergoes condensation to form acetoacetyl coenzyme A. The resulting compound then undergoes aldol condensation with acetyl coenzyme A to produce mevalonic acid. Mevalonic acid subsequently becomes dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), which are two precursors in sterol biosynthesis (Kanazawa 2001).

According to Faulkner (2000), Krasteva (2008), and Krueger (2006), sterols have various biological activities, including antifungal, antibacterial, anticancer, antimalarial, antimutagenic, anticholesterol, antidiabetic, and immunomodulatory properties.

Conclusion

Based on the macrophage assay results and spectroscopic data analysis, the following conclusions can be drawn:

The immunomodulator test results show that fraction FrKl3.1.3.1 has higher activity compared to FrKl3.1.3.2.

Spectroscopic data analysis reveals that UV-VIS exhibits maximum absorption at a wavelength (λmax) of 240 nm. The FTIR data indicate the presence of hydroxyl (-OH) groups, alkenes (double bonds, C=C), and aliphatic C-H bonds. The 1H-NMR data (CDCl3, 500 MHz) show the presence of six methyl groups, ten aliphatic methylene groups (-CH2-), nine geminal methylene groups with one methylene and one double bond, while the 13C-NMR and DEPT 135 data (CDCl3, 125 MHz) indicate that fraction *A. planci* FrKl3.1.3.1 consists of 28 carbon atoms, including six methyl (-CH3) groups, ten methylene (-CH2) groups, and nine methine (-CH-) groups.

Spectroscopic analysis data indicate that the compound in the chloroform fraction of *A. planci* FrKl3.1.3.1 is ergost-7-en-3-ol.

Acknowledgements

This research was supported by the Faculty of Fisheries and Marine Science, University of Khairun Ternate INDONESIA. Thanks to the Animal and Plant Taxonomy Laboratory, Faculty of Biology and Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia for providing Vero cells.

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