

# CHARACTERIZATION OF SECONDARY METABOLITES IN THE DICHLOROMETHANE FRACTION OF BLACK SEA CUCUMBER (*Holothuria atra*) FROM THE WATERS OF LEMUKUTAN ISLAND, WEST KALIMANTAN

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## ABSTRACT

The black sea cucumber (*Holothuria atra*) is a marine organism from the family Holothuridae that serves as a source of raw material for medicinal development and is abundant in the waters around Lemukutan Island, West Kalimantan. This study aims to isolate and identify secondary metabolites from the dichloromethane fraction of black sea cucumber from Lemukutan Island using FTIR and <sup>1</sup>H-NMR. The steps involved in this study include extraction (maceration and partitioning), phytochemical testing, fractionation (column chromatography), and the isolation and identification of molecular structures. Phytochemical testing of the dichloromethane fraction of *H. atra* revealed the presence of secondary metabolite compounds belonging to the saponin, phenolic, and alkaloid groups. Characterization of the compounds in the dichloromethane fraction using FTIR revealed peaks at wavenumbers 3376.54 cm<sup>-1</sup> (-OH stretch), 2855.38–2955.44 cm<sup>-1</sup> (-CH aliphatic), 1730.81 cm<sup>-1</sup> (C=O), 1635.43 cm<sup>-1</sup> (C=C), 1463.99 cm<sup>-1</sup> (C-H methylene), 1126.14 cm<sup>-1</sup> (C-O), and 1053.64 cm<sup>-1</sup> (C-C). The results of the isolate identification using <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) shows the presence of peaks at chemical shifts (δ) 0.68 ppm (1H, t), 0.84 ppm (9H, m), 1.19–1.24 ppm (22H, m), 1.44 ppm (1H, m), 1.55 ppm (3H, m), 1.95 ppm (1H, s), 4.09 ppm (41H, s), 7.07 ppm (1H, d, J=8.15 Hz), and 7.68 ppm (1H, d, J=6.9 Hz), which are characteristic of bis(2-ethylhexyl) 3-hydroxy-6-methyl phthalate compounds.

**Keywords:** Dichloromethane Fraction; Secondary Metabolites; *Holothuria atra*

## INTRODUCTION

Indonesia is a maritime nation with abundant biodiversity. According to Decree of the Minister of Marine Affairs and Fisheries No. 19 of 2022, the potential of Indonesia's marine biological resources amounts to 12,011,125 tons per year, comprising various animal species such as pelagic fish, shrimp, demersal fish, marine mollusks, sea cucumbers, food fish, ornamental fish, sea turtles, and seaweed (Anugrah & Alfaziri, 2021). One marine animal species widely used by the community is the sea cucumber. Sea cucumbers possess medicinal potential and can therefore be utilized in the health sector. Putram *et al.* (2017) reported that the sea cucumber *H. atra* exhibits anticancer activity due to bioactive compounds that inhibit cancer cell growth. Sea cucumbers are one of the fisheries commodities with high economic value. According to data from the Ministry of Marine Affairs and Fisheries, the export value of sea cucumbers increased from 2012 to 2016, ranging from approximately 905.2 to 2,003 tons per year. Additionally, according to data from the Central Statistics Agency, sea cucumber production from January to July 2019 amounted to 780 tons. Despite their high economic value and medical potential, the utilization of sea cucumbers as a fisheries resource in Indonesia remains relatively limited. It has not yet been optimized, particularly regarding the development of their bioactive compounds (Sasongko, 2020).

Indonesia has at least 56 sea cucumber species traded, comprising the genera *Actinopygea*, *Bohadschia*, *Holothuria*, *Pearsonothuria*, *Thelonata*, and *Stichopus* (Setyastuti *et al.*,

2019). The black sea cucumber (*H. atra*) is one of the sea cucumber species widely distributed in Indonesian waters; however, it has not yet been optimally utilized by the community. *Holothuria atra* is commonly known as the black sea cucumber or the keling sea cucumber and can thrive at various depths and in diverse habitats (Hartati *et al.*, 2017).

The black sea cucumber (*H. atra*) contains bioactive compounds with potential as antibacterial and antifungal agents. Research by Sukmiwati *et al.* (2019) indicates that *H. atra* contains bioactive compounds, including phenolics, terpenoids, and saponins. Putram *et al.* (2017) reported that the crude extract of *H. atra* contains alkaloids, steroid-triterpenoids, phenolics, and saponins. Additionally, research conducted by Yuliana *et al.* (2022) showed that the crude extract of *H. atra* contains phenolics, saponins, alkaloids, and terpenoids.

The crude extract of *H. atra* is rich in polar compounds such as phenolics, which exhibit antibacterial, anti-inflammatory, and antioxidant activities due to the presence of -OH and -OR groups (Yuliana *et al.*, 2022). The compounds in the crude extract of *H. atra* can be separated by polarity-based fractionation. Santos *et al.* (2015) stated that the dichloromethane fraction of sea cucumber has the highest potential to inhibit cancer cell growth. Research by Vaseghi *et al.* (2018) also showed that the dichloromethane fraction of sea cucumber (*Holothuria leucospilota*) exhibited the highest cytotoxicity against human umbilical vein endothelial cells (HUVEC). Other studies have also shown that the dichloromethane extract from the sea cucumber *H. leucospilota* possesses antioxidant and anti-proliferative activity against

cancer cell growth (Soltani & Baharara, 2014). However, studies on the secondary metabolites of the black sea cucumber from Lemukutan Waters, West Kalimantan, have not yet been reported. Therefore, this study aims to characterize the secondary metabolites of the dichloromethane fraction of the black sea cucumber (*H. atra*) collected from Lemukutan Waters, West Kalimantan.

## RESEARCH METHODS

### Research Location and Time

This study was conducted from January to October 2024 at the Chemistry Laboratory of the Faculty of Mathematics and Natural Sciences, Tanjungpura University. <sup>1</sup>H-NMR spectroscopy (500 MHz) was performed at the Chemistry Laboratory of the Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology. This study employed extraction, fractionation, and purification methods for the isolates as described by Yuliana *et al.* (2017).

### Equipment and Materials

The equipment used in this study included an aerator, Petri dishes, a Thin-Layer Chromatography (TLC) chamber, a glass funnel, a separating funnel, a distiller, a hot plate, needles, label paper, filter paper, clamps, a glass column for Flash Column Chromatography (FCC), UV lamps at 254 nm and 366 nm, a rotary vacuum evaporator (Heidolph), Fourier Transform Infrared Spectroscopy (FTIR) (Bruker Alpha II), nuclear magnetic resonance spectroscopy (<sup>1</sup>H-NMR: CDCl<sub>3</sub>, 500 MHz), and an analytical balance.

The materials used in this study were distilled water (H<sub>2</sub>O), 2N hydrochloric acid (HCl), sulfuric acid (H<sub>2</sub>SO<sub>4</sub>), anhydrous acetic acid ((CH<sub>3</sub>CO)<sub>2</sub>O), iron(III) chloride (FeCl<sub>3</sub>) 5%, dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), 96% ethanol (C<sub>2</sub>H<sub>5</sub>OH), ethyl acetate (C<sub>4</sub>H<sub>8</sub>O<sub>2</sub>), chloroform (CHCl<sub>3</sub>), methanol (CH<sub>3</sub>OH), n-hexane (C<sub>6</sub>H<sub>14</sub>), reagents (Dragendorff, Mayer, Wagner), silica G60 TLC plates F<sub>254</sub> (Merck), cerium(IV) sulfate (CeSO<sub>4</sub>), silica gel G60 (0.2–0.5 mm) (Merck), silica gel G60 (0.063–0.2 mm) (Merck), and black sea cucumber.

### Sample Preparation

The sample used in this study was the black sea cucumber *H. atra*, obtained from the waters around Lemukutan Island, Bengkayang Regency, West Kalimantan, at coordinates 0°46'30"N and 108°42'33"E. The *H. atra* sea cucumber samples were air-dried to a approximately 20% moisture content. The prepared sea cucumber samples were cleaned of any remaining sand and then weighed in their wet state to determine their weight. A total of 800 g of sea cucumber was cut into small, cube-shaped pieces approximately 0.5 × 0.5 cm for maceration.

### Extraction and Partition

The sea cucumber samples were extracted using 96% ethanol at a 1:2 (w/v) ratio, or approximately 1.6 liters of solvent for 800 grams of sample. The maceration process lasted 5 days, and the solvent was replaced daily by decantation. The extracted compounds were then evaporated at 40°C.

The resulting 44 grams of sea cucumber ethanol extract was then partitioned using dichloromethane at a volume ratio of extract: solvent (1:1.5) (v/v). The separatory funnel containing the sample was shaken and allowed to stand until

two layers formed. The dichloromethane layer was collected, and an equal volume of dichloromethane solvent was added back into the separating funnel. The resulting dichloromethane layer was then evaporated to obtain a concentrated dichloromethane fraction.

### Phytochemical Test

Phytochemical tests were conducted according to the methods described by Yuliana *et al.* (2022).

#### Alkaloid Test

An appropriate amount of the dichloromethane fraction was taken and dissolved in ethanol, then pipetted in 1 mL portions into three separate test tubes. Meyer's reagent, Dragendorff's reagent, and Wagner's reagent were then added to each tube in 5–10 drops.

#### Phenolic Test

One mL of the dissolved dichloromethane fraction was pipetted into a test tube, and 5–10 drops of FeCl<sub>3</sub> solution were added.

#### Flavonoid Test

Pipette 1 mL of the dissolved dichloromethane fraction into a test tube. Add magnesium (Mg) to the tube, then add 5–10 drops of HCl.

#### Saponin Test

One mL of the dissolved dichloromethane fraction was pipetted into a test tube, followed by the addition of 1 mL of heated distilled water (H<sub>2</sub>O). The tube was shaken until foam formed. The test result is positive if the foam remains stable for 15 minutes after the addition of a few drops of 2N HCl.

#### Steroid or Triterpenoid Test

Pipette 1 mL of the dissolved dichloromethane fraction into a test tube, then add 5–10 drops of Liebermann-Burchard reagent.

### Isolation and Purification of Compounds

The dichloromethane fraction was separated through thin-layer chromatography, flash column chromatography, and preparative thin-layer chromatography. The dichloromethane fraction was separated using pressure column chromatography (PCC) with a gradient eluent. The eluents used were 100% n-hexane, n-hexane: dichloromethane (8:2), 100% dichloromethane, dichloromethane: ethyl acetate (90:10), 100% ethyl acetate, and 100% methanol. Silica gel was packed wet into a 5 cm column and allowed to stand for 24 hours until the silica solidified to a height of approximately 15 cm. Elution was assisted by an aerator to accelerate eluent flow. The eluate was collected into pre-weighed numbered 10 mL vials.

The Thin-layer Chromatography (TLC) then identified the resulting eluates based on their separation patterns. TLC was performed on a 5×5 cm silica plate with upper and lower margins of 0.5 cm and 1 cm, respectively. The eluted plates were then examined for spot patterns under UV light at 254 nm and 366 nm, followed by spraying with a cerium(IV) sulfate spot-revealing reagent. Eluates with the same separation patterns were combined to yield 5 pooled fractions. The masses of these pooled fractions were determined, and the pooled fraction to be purified was selected.

The selected fraction is separated using preparative thin-layer chromatography (TLC). The TLC begins by preparing a 20×10 cm silica plate and a TLC chamber of appropriate size. The combined fraction is spotted along the bottom edge of the silica plate and eluted using an ethyl acetate: methanol (97:3) eluent. The eluted TLC plate is then observed under UV light at 366 nm and 256 nm. TLC subsequently tests the resulting isolate to assess its purity.

The two-dimensional TLC purity test begins by preparing a 5×5 cm TLC plate. The isolate's purity is assessed using two-dimensional TLC, with the first elution using an ethyl acetate: methanol (9.4:0.6) eluent. The second elution was performed by rotating the plate 90° and eluting with an ethyl acetate: methanol (9.6:0.4) mixture. The eluted TLC plate was then observed under UV light at 366 nm and 256 nm.

### Compound Characterization

The functional group analysis of the dichloromethane fraction of *H. atra* was performed using a *Fourier Transform Infrared Spectroscopy* (FTIR) instrument. The molecular structure of the isolated compound was analyzed using a *Nuclear Magnetic Resonance* (NMR) spectrometer (<sup>1</sup>H-NMR: CDCl<sub>3</sub>, 500 MHz).

## RESULTS AND DISCUSSION

The *H. atra* was extracted with ethanol until the entire sample was submerged. This maceration process aimed to extract the compounds contained within the sea cucumber. The maceration process was carried out for 5 days, with solvent replacement every 24 hours, until a clear filtrate was obtained. This clear filtrate indicated that the majority of the active compounds within the sea cucumber had been maximally extracted. The extracted sample was decanted to separate the filtrate from the solid residue. The filtrate was concentrated using a rotary evaporator at 40°C until a thick, paste-like extract formed. The resulting black sea cucumber (*H. atra*) ethanol extract weighed 44 grams and had a dark (brick-red) color. Research conducted by Oktaviani *et al.* (2015) indicates that the brick-red color of this sea cucumber ethanol extract is due to the presence of triterpenoid compounds.



**Figure 1.** Ethanol Extract of Black Sea Cucumber (*H. atra*)

The ethanol extract of *H. atra* was then subjected to liquid-liquid fractionation using Dichloromethane as the solvent at an extract: solvent ratio of 1:1.5. Dichloromethane was selected as a semipolar solvent to separate semipolar to nonpolar compounds present in the ethanol extract, such as triterpenoids and steroids. Dichloromethane has a density of 1.326 g/mL, so its layer will be at the bottom of the separation funnel (Obenu, 2019).

The dichloromethane layer was collected and concentrated using a rotary evaporator at 40°C until a thick, paste-like extract formed. The resulting dichloromethane fraction weighed 2.2379 grams and was brick-red. Santos *et al.* (2015) reported that the dichloromethane fraction from sea cucumbers exhibits the highest potential to inhibit cancer cell growth. Other studies also indicate that the dichloromethane extract from the sea cucumber *H. leucospilota* exhibits antioxidant and anti-proliferative activity against cancer cell growth (Soltani & Baharara, 2014). A comparison of the sea cucumber mass obtained from the extraction process through partitioning is presented in Table 1.

### Phytochemical Analysis

The results of the identification of secondary metabolites in the ethanol extract and dichloromethane fraction from black sea cucumber (*H. atra*) samples originating from the waters of Lemukutan Island, West Kalimantan, are shown in Table 2.

**Table 1.** Sample Yield Results

Sample Description	Weight (g)	Yield (%)
Whole sea cucumber	800	-
Ethanol extract	44	5.5
n-Hexane fraction	1.3045	2.95
Dichloromethane fraction	2.2379	5.08

**Table 2.** Phytochemical Test Results of the Ethanol Extract and Dichloromethane (DCM) Fraction of *H. atra*

Phytochemical test	Reagent	Ethanol extract	n-Hexane Fraction	DCM Fraction
Flavonoid test	Mg + concentrated HCl	-	-	-
Phenolic test	FeCl <sub>3</sub> 5% Water +	+++	+	+
Saponin test	HCl	+++	+++	+++
Alkaloid test	Mayer	+	+	+++
	Wagner	+	+++	+++
	Dragendorff	+++	+++	+++
Steroid test	Liebermann-Burchard	+	+++	-
	Liebermann-Burchard Jalkowski	-	-	-

Legend: +++ = formation of precipitate; += color change; -= not detected

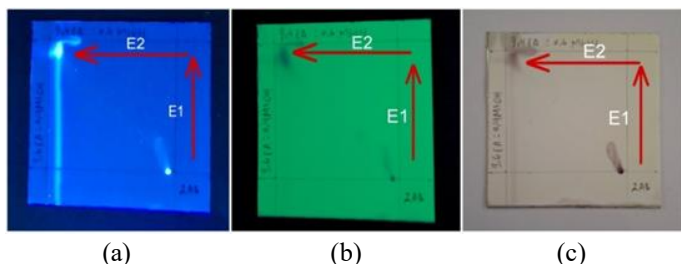
Notes: +++ = formation of precipitate; += color change; -= not detected

The results of the phytochemical analysis of secondary metabolites in Table 2 show that the ethanol extract of *H. atra* contains phenolic compounds, saponins, alkaloids, and steroids. At the same time, the DCM fraction was identified to contain phenolic compounds, saponins, and alkaloids. These results are consistent with studies by Yuliana *et al.* (2022) and Nursid *et al.* (2019); however, the flavonoid and terpenoid tests yielded positive results in those studies.

### Isolation and Purification of Compounds

A 1.2-gram portion of the dichloromethane fraction was impregnated using silica gel 60 (0.2–0.5 mm) in a 1:1 (w/w) ratio to ensure optimal sample separation (Jayanti *et al.*, 2012). A total of 71.8 grams of silica gel was packed wet into a 5 cm column and left to stand for 24 hours until the silica solidified to a height of approximately 15 cm. Elution in the column was performed using a gradient method, where the eluents used were 100% n-hexane, n-hexane: dichloromethane (8:2), 100% dichloromethane, dichloromethane: ethyl acetate (90:10), 100% ethyl acetate, and 100% methanol. The mobile phase is passed through the column, allowing the compound bands to move at different speeds depending on their properties. The eluates are collected in sequentially numbered 10 mL vials, resulting in 274 vials.

The obtained fractions were analyzed using TLC to examine the compound spot patterns in each vial. This process was conducted by selecting 1 vial to represent 3 vials. The TLC process utilized a gradient eluent system with increasing polarity to align with the separation patterns observed in each fraction. The first eluents used were n-hexane: dichloromethane (5:5), (4:6), and (3:7). The separation patterns on the TLC plates were observed under UV light at 254 nm and 366 nm, and the plates were sprayed with a spot-revealing reagent containing cerium sulfate. The relatively similar TLC chromatogram patterns were combined, yielding 5 groups of fractions (vial codes: *Holothuria atra*-Dichloromethane Fraction 1 (HA-FD1) – *Holothuria atra*-Dichloromethane Fraction 5 (HA-FD5)). Based on the elution results of the combined fractions using TLC plates and the characteristics of the fraction with the highest mass and largest spot, the HA-FD5 fraction was analyzed using an FTIR instrument. This fraction was then separated by preparative thin-layer chromatography (P-TLC) using an ethyl acetate:methanol (97:3) eluent.



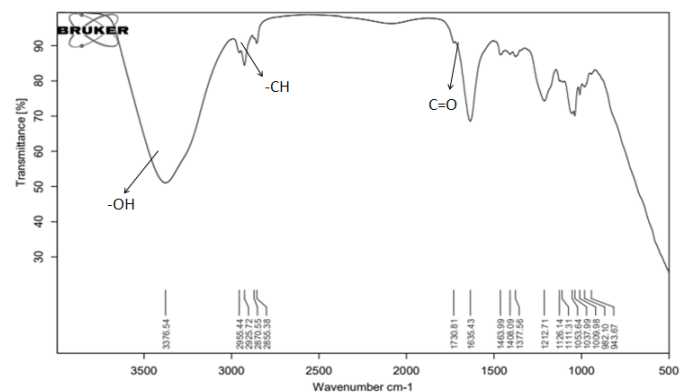
**Figure 2.** Two-dimensional TLC results of isolate 2AB under 366 nm UV light (a), 254 nm UV light (b), and cerium staining (c)

The TLC results for the HA-FD5 fraction showed 5 distinct spots visible under UV light at 254 and 366 nm. The resulting spots were then marked with a pencil under UV light to facilitate scraping. The scraped silica was collected into separate vials, then soaked in methanol and filtered using Whatman filter paper. The obtained isolates were analyzed by TLC, and isolate 2 (2AB), weighing 14.4 mg, was selected based on its R<sub>f</sub> value. The purity of isolate 2AB was tested by two-dimensional TLC, with the first elution using ethyl acetate: methanol (9.6:0.4) and the second using ethyl acetate: methanol (9.4:0.6), which is more polar.

The purity test results using two-dimensional TLC showed that isolate 2AB was relatively pure, with slightly glowing spots and stains remaining at the initial spotting points, indicating the presence of impurities. The isolate was subsequently analyzed by nuclear magnetic resonance spectroscopy (<sup>1</sup>H-NMR) to determine its molecular structure.

### Characterization of Compounds Using FTIR Spectroscopy

Structural analysis using FTIR aimed to identify the functional groups present in the sample (HA-FD5 fraction). The analysis was performed using a Bruker FTIR spectrometer. The FTIR spectrum obtained from the HA-FD5 fraction is shown in Figure 3. The functional groups identified in the sample are presented in Table 3.



**Figure 3.** FTIR spectrum of DCM fraction 5

The infrared (IR) identification results for DCM fraction-5 show an absorption peak at 3376.54 cm<sup>-1</sup>, indicating a hydrogen bond (OH). Wavenumbers appearing in the 3200–3400 cm<sup>-1</sup> range indicate the presence of hydroxyl groups from –OH stretching (Nandiyanto *et al.*, 2019). Absorption peaks at wavenumbers 2955.44 cm<sup>-1</sup>, 2925.72 cm<sup>-1</sup>, 2870.55 cm<sup>-1</sup>, and 2855.38 cm<sup>-1</sup> indicate the presence of aliphatic –CH stretching. The vibrational peak at 1730.81 cm<sup>-1</sup> indicates the presence of the carbonyl C=O functional group from the ester. This is supported by the findings of Nandiyanto *et al.* (2019), who reported that the wavenumber range of 1750–1700 cm<sup>-1</sup> indicates the presence of carbonyl groups such as ketones, aldehydes, carboxylic acids, or esters. The peak at 1635.43 cm<sup>-1</sup> indicates the presence of an aromatic C=C group. Wavenumbers in the 1650–1600 cm<sup>-1</sup> range indicate the presence of double bonds or aromatic components. The absorption peak at a wavenumber of 1463.99 cm<sup>-1</sup> indicates the presence of a methylene C-H bond (CH<sub>2</sub>), which is consistent with the literature, where the peak of the methylene C-H bond functional group appears in the wavenumber range of 1485–1445 cm<sup>-1</sup> (Nandiyanto *et al.*, 2019). The next peak is observed at 1126.14 cm<sup>-1</sup>, indicating stretching of the C-O ester group. The peak at 1053.64 cm<sup>-1</sup> indicates C-C vibrations in the aliphatic chain. According to Nandiyanto *et al.* (2019), the peaks in the wavenumber range of 1300–700 cm<sup>-1</sup> arise from C–C group vibrations. The next peak occurs at 1037.99 cm<sup>-1</sup>, indicating the presence of a C–O–C functional group.

**Table 3.** Functional Group Analysis Results of DCM Fraction 5

Wavenumber of HA-FD5 (cm <sup>-1</sup> )	Wavenumber Range (cm <sup>-1</sup> )				Predicted functional group
	Sharifi <i>et al.</i> , (2017)	Jegade <i>et al.</i> , (2019)	Sanna <i>et al.</i> , (2015)	Javed <i>et al.</i> , (2022)	
-	742	-	-	-	(CH <sub>2</sub> ) <sub>n</sub> -rocking
1037.99	-	-	-	1039.26	C-O-C
1053.64	-	1040.04	-	-	C-C (aliphatic)
-	-	-	-	1070.24	C-O stretch (ester)
1126.14	1122	1123.01	-	-	-
-	-	-	-	1460.99	C=C (aromatic)
1463.99	1464	-	-	-	C-H (methylene)
1635.43	-	1600.24	-	1599.75	C=C (aromatic)
1730.81	1728	1738.01	1730	1723.37	C=O (carbonyl)
2855.38	2860	-	2858	2859.60	-
2870.55	-	-	2875	-	C-H stretch (alkane)
2,925.72	-	-	2925	-	-
2,955.44	2956	2,959.27	2962	-	-
-	-	3070.98	-	-	C-H (aromatic)
3,376.54	-	-	-	-	-OH/H <sub>2</sub> O

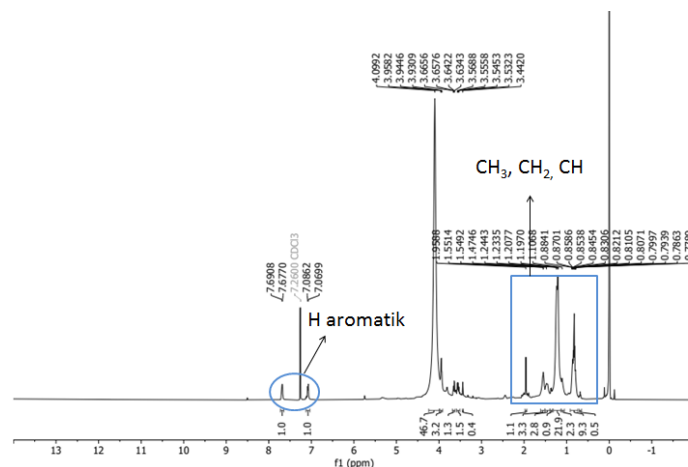
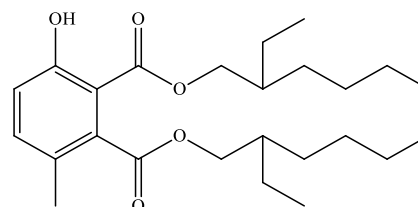
### Characterization of Compounds Using Nuclear Magnetic Resonance Spectroscopy (<sup>1</sup>H-NMR)

The FD5-AR2AB isolate was identified using a Nuclear Magnetic Resonance (NMR) spectrometer. The identification was performed by <sup>1</sup>H-NMR using deuterated chloroform (CDCl<sub>3</sub>) as the solvent at 500 MHz. The spectrum obtained from the FD5-AR2AB isolate is shown in Figure 4.

The <sup>1</sup>H-NMR spectrum of the isolate shows two signals from aromatic hydrogen atoms at 7.68 ppm (d, J = 6.9 Hz) and 7.07 ppm (d, J = 8.15 Hz), indicating that this compound contains a benzene ring (Sharifi *et al.*, 2017). The proton signals at 1.19–1.24 (m) and 1.44 (m) are characteristic of the methylene group (CH<sub>2</sub>). The proton signal at a chemical shift of 4.09 ppm indicates the presence of a hydrogen atom from the hydroxyl group (OH). The proton signals at 0.84 ppm (m) and 0.68 ppm (t) are from H atoms of the methyl group bound to the methylene (CH<sub>3</sub>-CH<sub>2</sub>-) (Dotulong *et al.*, 2022). The interpretation of the <sup>1</sup>H-NMR spectrum of the isolate indicates a similarity to the structure of bis-(2-ethylhexyl) phthalate (DEHP), but modified with hydroxyl (OH) and methyl (CH<sub>3</sub>) groups, as described by Yuan & Cheng (2017). The proposed structure of the compound is shown in Figure 5.

Previous research conducted by Sharifi *et al.* (2017) showed that phthalate compounds were also found in the sea cucumber *H. atra* from the Persian Gulf, Iran. The phthalate compound identified was bis(2-ethylhexyl) phthalate (DEHP). Another study by Keshavarz *et al.* (2021) also showed that *Holothuria parva* contains diisooctyl phthalate. Soliman *et al.* (2016) also reported the presence of phthalate compounds in the extract of the sea cucumber *H. atra*, specifically phthalic acid and 4-bromophenyl ethyl ester. Additionally, a study by Muslim *et al.* (2023) identified 2-ethyl-1-hydroxyhexyl (2-ethylhexyl) phthalate in the n-hexane fraction of the *Aaptos suberitoides* sponge collected from waters around Lemukutan Island, West Kalimantan. The presence of phthalate compounds in marine biota is due to microplastic pollution in

the water. Fadhilah *et al.* (2023) conducted a study on microplastic abundance in the waters around Temajo Island, West Kalimantan. The study showed that microplastics contaminated the waters around Temajo Island with an abundance of 6,823.30 particles/L. This water pollution can contaminate marine biota and threaten the biodiversity of both plants and animals in surrounding waters.

**Figure 4.** <sup>1</sup>H-NMR spectrum of isolate FD5-AR2AB**Figure 5.** Proposed Structure of Bis(2-ethylhexyl) 3-hydroxy-6-methyl phthalate

**Table 4.** <sup>1</sup>H-NMR Spectrum Data of Isolate FD5-AR2AB

H Position	Chemical Shift (δ ppm)				
	(Kdimy <i>et al.</i> , 2023)	Bis(2-ethylhexyl) phthalate (Kdimy <i>et al.</i> , 2023)	Di(2-ethylhexyl) phthalate (DEHP) (Genay <i>et al.</i> , 2016)	DEHP (Yuan <i>and</i> Cheng, 2017)	Isolate FD5-AR2AB
1 (-OH)	-	-	-	-	4.09 (s), 41H
2	7.66–7.61 (m), 2H	7.68–7.58 (m)	7.53 (dd), 2H	7.00 (d) -H	7.68 (d), 1H (6.9 Hz)
3	7.66–7.61 (m), 2H	7.68–7.58 (m)	7.53 (dd), 2H	7.00 (d) -H	7.07 (d), 1H (8.15 Hz)
4 (CH <sub>3</sub> )	-	-	-	-	1.95 (s), 1H
5.5'	4.28–4.18 (m), 4H	4.29–4.19 (m)	4.24 (dd), 2H	4.25–4.09 (m), 4H	4.09 (s), 41H
6.6'	1.69 (hept) (6.0 Hz), 2H	1.71 (hept) (6.0 Hz)	4.20 (dd), 2H	1.59–1.72 (m), 4H	1.55 (m), 3H
7.7'	1.39–1.32 (m)	1.43–1.30 (m)	1.68 (sept), 2H	1.26–1.44 (m), 4H	1.19–1.24 (m), 22H
8.8'	1.39–1.32 (m)	1.43–1.30 (m)	1.5–1.25 (m), 16H	1.26–1.44 (m), 4H	1.19–1.24 (m), 22H
9.9'	1.42–1.31 (m), 12H	1.43–1.30 (m)	1.5–1.25 (m),	1.26–1.44 (m), 4H	1.19–1.24 (m), 22H
10.10	1.00–0.90 (m)	1.00–0.91 (m)	0.92(t), 6H	0.86–0.95 (m), 6H	0.84 (m), 9H
11,11'	1.51–1.42 (m), 4H	1.46 (tdd) (7.2, 6.4, 3.3 Hz)	1.5–1.25 (m),	1.26–1.44 (m), 4H	1.44 (m), 1H
12.12'	1.00–0.90 (m), 12H	1.00–0.91 (m)	0.89 (t), 6H	0.86–0.95 (m), 6H	0.68 (t), 1H

## CONCLUSION

The dichloromethane fraction of the black sea cucumber (*H. atra*) contains secondary metabolites from the phenolic, saponin, and alkaloid groups. Compounds from the dichloromethane fraction identified using FTIR spectroscopy revealed the presence of functional groups, including hydroxyl groups from the –OH stretch, aliphatic –CH groups, and carbonyl groups C=O. Characterization of the isolate using <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>) revealed chemical shifts for aromatic protons (H), hydroxyl groups, as well as protons from methyl, methylene, and methine groups. Based on the interpretation of the FTIR and <sup>1</sup>H-NMR results, it is suspected that the FD5-AR2AB isolate is 3-hydroxy-6-methyl phthalate, and this compound bears a resemblance to the main framework of bis(2-ethylhexyl) phthalate.

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