Isolation of sea cucumbers’s (Holothuria atra) secondary metabolite using column-chromatography technique

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Abstract. Identification of sea cucumbers from Benteng Inong Balee, Aceh Besar and their phytochemistry screening were conducted in December 2020 to January 2021 at Laboratory of Marine Chemistry and Fisheries Biotechnology, Universitas Syiah Kuala. The purpose of this study was to identify the species of sea cucumbers and its secondary metabolite content using phytochemistry screening and column chromatography. The species of sea cucumbers that were identified was Holothuria atra. The extraction method used in sea cucumber extraction was maceration method, while the separation of secondary metabolites used column-chromatography with eluent of n-hexane : ethyl acetate (8:4). The results showed that secondary metabolites obtained from phytochemical tests were flavonoids, saponins and triterpenoids.

1. Introduction
Secondary metabolites are referred for the chemical compounds produced by organism and plants that serve them on several functions. Those are weapons used against predators, metal transporting agents, symbiosis agents, sexual hormones, and differentiation effectors [1]. Extraction and purification of secondary metabolites using particular solvent and method have proved that the secondary metabolized are promising utilized as antitumor, antimicrobial, antidiabetic, antioxidant, immuno-suppressants, cholesterol-lowering agents, wound healing, and anti-inflammatory [2, 3].

Secondary metabolites from marine organism, such as sponges, tunicates, corals, algae, and invertebrates, have been well reported [4-8]. As one of invertebrates group, sea cucumbers are potential marine organism in producing secondary metabolites. Indonesia has at least 26 species of sea cucumbers that have been recorded including Actinopyga echinites, A. lecanora, A. mauritiana, A. miliaris, Bohadschia argus, B. marmorata, Holothuria atra, H. coluber, H. edulis, H. fuscopunctata, H. fuscogilva, H. impatiens, H. leucospilota, H. nobilis, H. ocelata, H. pervicax, H. scabra, H. similis, Pearsonothuria graeffei, Stichopus chloronotus, S. horrens, S. variegatus, Thelenota ananas, T. anax [9]. In Aceh, it has been identified 5 species of sea cucumbers which are Teripang cera-cera, S. horren, H. argus, H. fuscogilva, and Teripang polos [10].
Previous studies of secondary metabolite isolation of sea cucumber have been conducted by phytochemical screening. The studies reported that different species may produce dissimilar secondary metabolite. For example, *A. miliaris* produces steroid and saponin as well as *B. argus* [11], *H. hilla* release flavonoid and saponin [12], *H. scabra* produced alkaloid, saponin, flavonoid, steroid, and triterpenoid [13-15], while *S. chloronotus* only contains of flavonoid [11]. However, all the secondary metabolite on those studies were analysed directly into phytochemical screening without using the purifying technique.

One of several techniques frequently applied in isolating and purifying the secondary metabolite is chromatography. Chromatography has been known as a method that gives a broad range of substances dissolved in all reagents and a recovery of solvent used [16]. Therefore, this research was conducted to isolate the secondary metabolites of sea cucumber collected from Benteng Inong Balee, Aceh Besar Regency using column-chromatography technique.

2. Material and Methods

2.1 Time and location

This research was conducted from December 2020 to February 2021. Samples were collected in Benteng Inong Balee, Lamreh Village, Mesjid Raya District, Aceh Besar Regency. All the procedures including the identification of cucumber compounds, secondary metabolite isolation and phytochemical screening were carried out at the Laboratory of Marine Chemistry and Fisheries Biotechnology, Faculty of Marine Affairs and Fisheries, Syiah Kuala University.

![Figure 1. Map of Research Location](image)

2.2 Procedures

2.2.1 Sea cucumber identification

Samples were obtained from Benteng Inong Balee, Lamreh Village, Mesjid Raya District, Aceh Besar District. The samples were collected by the visual census method with a length of 50 m and a width of 2 m. The depth of observation reaches 0.5-2 meters. The samples obtained were put into a cooling box. The samples were identified at the Marine Chemistry and Biotechnology Laboratory. Identification was carried out by morphological observations and matched with images in the Indonesian Sea Cucumber literature to determine the identification of these types of sea cucumbers [17, 18].
2.2.2 Sample extraction
Wet sea cucumber samples were extracted using methanol as a solvent with a sample ratio of 1:2 applying the maceration method. The solvent was changed and repeated up to 3 times during 24 hours. The samples were filtered with Whatman paper no. 41 to produce pulp and macerate. The macerate was put into the evaporator at a temperature of 40°C. The solvent was brought to completely evaporate until a thick extract was yielded [19].

2.2.3 Isolation using column-chromatography and Phytochemical screening
Isolation of secondary metabolite by column chromatography used a mixture of n-hexane : ethyl acetate (8:4) as a mobile phase and silica gel GF254 as a stationary phase. Firstly, the silica gel was suspended with the eluent and put into a column with cotton-plugged bottom for 24 hours. The extract was dissolved with a small amount of eluent and silica gel. Then, it was inserted into the column and eluted. The results of separation by column chromatography were placed in an Erlenmeyer flask.
Phytochemical screening was performed by using a specific reagent or chemicals to indicate the presence of secondary metabolite (Table 1).

| Compound tested | Reagents or chemicals | Presence indicators |
|-----------------|-----------------------|---------------------|
| Alkaloid        | Wagner’s reagent       | A reddish-brown precipitate |
| Flavonoid       | Mg powder and concentrated HCl | A colour changing into yellow or orange |
| Saponin         | Distilled water        | A foam layer forming  |
| Triterpenoid    | Liebermen-Burchard’s reagent | A brown ring was formed |
| Steroid         | Liebermen-Burchard’s reagent | A greenish blue ring was formed |

3. Result and Discussion
3.1 Sea cucumber identification
The identification of sea cucumbers collected from Benteng Inong Balee, Aceh Besar was carried out through a taxonomic test according to Setyastuti et. al. [17]. In this study, it was collected three individuals. According to the reference, the samples were identified as the found as Holothuria atra. The observations of as Holothuria atra are presented in Table 2.

| Characteristics | Observation |
|-----------------|-------------|
| Body shape      | Cylindrical elongated like a cucumber ranged in size from 10-40 cm, soft and fleshy body wall |
| Color           | Shiny black |
| Odor            | fishy       |
| Dorsal          | There is a soft spike are small and dense (papilla) |
| Ventral         | Filled premises tiny tube feet long and densely arranged |
| Posterior       | There is an anus (drain hole) |
| Anterior        | There is a mouth surrounded by a fringe (tentacles) |

3.2 Isolation using column-chromatography and Phytochemical screening
Phytochemical test of methanol extract stated that the Holoturia atra contained secondary metabolites including flavonoids, saponin, and triterpenoid (Table 3).

The presence of alkaloids in the extract of black sea cucumber (Holothuria atra) was not identified in the methanol extract. This was indicated by the absence of a reddish brown precipitate after the addition of Wagner's reagent. Alkaloids are cyclic compounds that do not have color and are easily decomposed by heat caused by its alkaline properties and polar alkaloids. Alkaloids are one of the secondary metabolites that are widely used as medicines. Alkaloids are compounds derived from amino acids that function as analgesics, anti-malarial, and regulate the work of the heart. Alkaloids also have effects as sedatives, raise blood pressure, antibacterial and nervous system triggers [20].
The presence of flavonoids in the extract of black sea cucumber (Holothuria atra) was identified in the methanol extract. This was indicated by the formation of a thick yellow color after adding 0.05 grams of Mg and 1 ml of concentrated HCl. Flavonoids are polar compounds that able to provide protection against free radicals. Flavonoids found in black sea cucumbers can be used as anti-inflammatory, antitumor, antiviral and antibiotic [21].

The presence of saponins in the extract of black sea cucumber (Holothuria atra) was identified in the methanol extract. It was characterized by the formation of foam along the 1.5 cm which lasts for 10 minutes. The nature of saponins can reduce the surface tension of water so that this can cause foam or foam when the extract is added to warmed distilled water. In the body of sea cucumbers, secondary metabolites are found that can be used as antibacterial [22].

The presence of triterpenoids in the extract of black sea cucumber (Holothuria atra) was identified in the methanol extract. This was indicated by the formation of a brown ring on the boundary of the solution when Lieberman Burchad's reagent was added. Lieberman Burchad reagent is a mixture of chloroform, acetic anhydride and sulfuric acid. Triterpenoid/steroid compounds would be dehydrated when sulfuric acid (H2SO4) was added and form ions that gave a color reaction. The color change in triterpenoid/steroid compounds occurs due to an oxidation reaction so that a conjugated double bond is formed. Triterpenoids function as diabetes drugs, malaria drugs, anti-inflammatory, leukemia and allergy drugs [23].

4. Conclusion
The type of sea cucumber found in Benteng Inong Balee was black sea cucumber (Holothuria atra). Compound of secondary metabolites contained in the methanol extract and purified by column-chromatography using a mixture of n-hexane:ethyl acetate (8:4) namely flavonoids, saponins, and triterpenoids.

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