Pharmacogn. Res.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcogres.com | www.phcog.net

ORIGINAL ARTICLE

Cytotoxicity and Apoptosis Induction of Sea Cucumber *Holothuria atra* Extracts

Muhammad Nursid, Endar Marraskuranto, Ekowati Chasanah

Research Center for Marine and Fisheries Products Processing and Biotechnology, Ministry of Marine Fisheries and Affairs, Jakarta, Indonesia

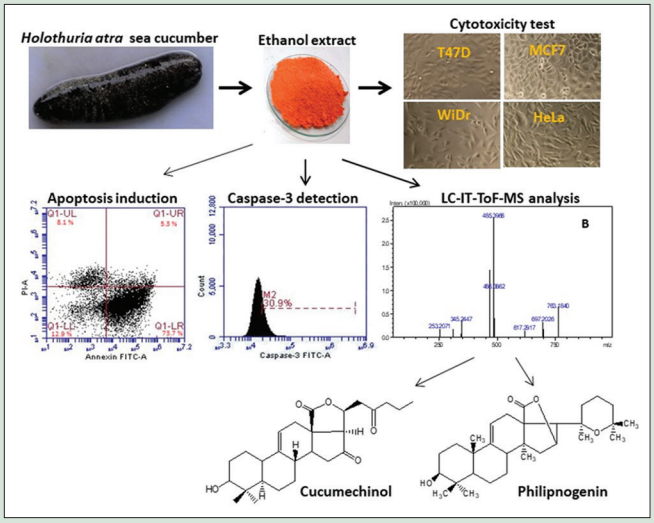
ABSTRACT

Context: Cancer is one of the major causes of death. Sea cucumbers are marine invertebrate that is widely used and utilized in traditional medicine. One of the most studied of sea cucumbers efficacy is its anticancer property. The anticancer property of sea cucumbers is much related to the content of an active compound called saponin. **Objective:** The objective of this research is to determine the cytotoxicity and apoptosis induction of sea cucumber *Holothuria atra* ethanol extract and to study its anticancer active compound. **Materials and Methods:** Sea cucumber of *H. atra* was taken from Halmahera waters, North Maluku Indonesia. The extraction was conducted by maceration method using ethanol 96% then continued with liquid-liquid partition and separation using C18 column. The investigation of active compound content was conducted by phytochemical and spectroscopic analysis using liquid chromatography-ion trap-time of a flight-mass spectrophotometer (LC-IT-ToF-MS). The cytotoxicity test was performed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide method against human breast ductal carcinoma cell (T47D), human breast adenocarcinoma (MCF7), human colorectal adenocarcinoma cell (WiDr), and human cervix adenocarcinoma cell lines (HeLa). The apoptotic induction test was performed using flow cytometry using Fluorescein isothiocyanate (FITC) Annexin-V-Apoptosis and caspase-3 detection using FITC Active Caspase-3 kit. **Results:** Phytochemical test showed that ethanol extract of *H. atra* contained alkaloids, flavonoids, steroids-triterpenoids, phenols, and saponins. The extract showed cytotoxic activity against the 4 cell lines with Inhibition concentration 50 values ranging from 9.6 to 14.3 µg/ml. Flow cytometry analysis showed that the T47D cell population underwent apoptosis after treated with ethanol extract. The extract also activated caspase-3 on the T47D cells. The results of LC-IT-ToF-MS analysis showed that the active fraction from C18 column separation contained saponin and identified as Cucumechinol and Philinopgenin B. **Conclusions:** The results of this study indicated that *H. atra* active extract has good cytotoxicity and has potential to be developed as an anticancer agent.

Key words: Apoptosis, caspase-3, cytotoxicity, *Holothuria atra*, saponin, sea cucumber

SUMMARY

- Ethanol extract of sea cucumber *Holothuria atra* from the Halmahera Waters Indonesia exhibited cytotoxicity against several cancer cell lines. The extract was able to induce apoptosis on T47D cell. Base on mass spectra analysis, cucumecihnol and philipnogenin were responsible for the bioactivity,



Abbreviations used: MNPs: Marine natural products; LC-IT-ToF-MS: Liquid chromatography-ion trap-time of flight-mass spectrophotometer; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; T47D: Human breast ductal carcinoma cell; MCF7: Human breast adenocarcinoma cell; WiDr: Human colorectal adenocarcinoma cell; HeLa: Human cervix adenocarcinoma cell; RPMI: Roswell park memorial institute medium; FBS: Fetal bovine serum; FITC: Fluorescein isothiocyanate; PBS: Phosphate buffer saline; BD: Becton Dickinson; IC_{50}: Inhibition concentration 50; PS: Phosphatidylserine; PI: Propidium iodide.

Correspondence:
Dr. Muhammad Nursid,
KS. Tubun Petamburan VI Street, Jakarta, 10260, Indonesia.
E-mail: muhammadnursid@gmail.com

DOI: 10.4103/pr.pr_3_18

Access this article online
Website: www.phcogres.com
Quick Response Code:

INTRODUCTION

Cancer is one of the major causes of death in addition to cardiovascular disease. According to the American Cancer Society,[1] the most common cancer is colon cancer (colorectal), prostate, and lung cancer (lung cancer). Breast cancer is the most common cancer in women while in men is lung cancer. Colorectal cancer ranks second in both men and women. The search for anticancer drug research is now largely targeted to the molecular mechanisms involved in the cell cycle and the process of apoptosis in cancer cells. There is strong evidence that the growth of cancer is not only due to the unlimited proliferation of cells but also by the occurrence of defects in the apoptotic process.[2] Apoptosis is an intrinsic death program in cells and plays an important role in physiological processes during immune system maturation, embryogenesis, and tissue exchange.[3] The main control mechanism if a damaged DNA cell fails to be repaired is through apoptosis. Many malignancy processes are characterized by defects in

Cite this article as: Nursid M, Marraskuranto E, Chasanah E. Cytotoxicity and apoptosis induction of sea cucumber *Holothuria atra* extracts. Phcog Res 2019;11:41-6.

apoptotic regulatory pathways.[4] Therefore, studies conducted to search drug compounds that work specifically against certain molecules that can trigger apoptosis in cancer cells is in urgent needs.[5,6] One of the challenges that arise in developing anticancer drugs is the presence of cancer cell which is resistance either to cytotoxic agents or to proapoptosis signals.[7]

The ocean covering over 70% of the Earth's surface is a source of organisms capable of producing bioactive secondary metabolites with a unique structure often called marine natural products (MNPs).[8] In recent years, MNPs have attracted the attention of both chemists and pharmacologists due to their chemical diversity and bioactivity,[9] one of them is for anticancer drug compounds. To date, there are five compounds of marine invertebrates already approved for use in cancer therapy, i.e., cytarabine (from sponge), ziconotide (from cone snail), trabectedin (from tunicate), eribulin mesylate (from sponge), and brentuximab vedotin (from sponge).[7]

One of the potential marine invertebrates used as a producer of anticancer compounds is sea cucumber. Sea cucumber is a member of the *Holothuroidea* which is included in the phylum Echinodermata. This group of animals has a cucumber-shaped like and lives in the sea. This type of *Holothuroidea* is estimated to be more than 1200 species in the world, but only 66 species are considered as sea cucumber.[10] Setyastuti and Purwati[11] reported that there are about 350 species of *Holothuroidea* where about 54 species are grouped as a sea cucumber in Indonesia. Sea cucumber is one type of marine invertebrate that attracts researchers and nutritionists because it has many health benefits and is now widely used in the treatment of chronic inflammatory diseases.[12] It is widely studied for its efficacy as anti-inflammatory, immunostimulant, and cancer prevention and treatment. Until now, pharmacological bioactive compounds from sea cucumbers have been identified, including antiangiogenic bioactive compounds, anticancer, anticoagulant, antihypertension, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, and as a wound healer. Some types of the bioactive compounds that are responsible for their bioactivity are triterpene glycosides, chondroitin sulfates, glycosaminoglycans, polysaccharides sulfate, sterols, phenolics, peptides, cerebrosides, and lectins.[13,14]

Holothuria atra is one of sea cucumber species which is abundant in Indonesian waters. Research on *H. atra* as the source of anticancer compounds is still limited. Compared to the other highly economically value sea cucumber i. e. *H. scabra* and *Stichopus variegatus*. *H. atra* is yet widely to be utilized. It might have to do with the limited information on natural compounds content in *H. atra* that are pharmacologically active such as anticancer. Some studies on active extracts from *H. atra* were revealed by Dhinakaran and Lipton[15,16] and Murniasih *et al*.[17] Methanol extract from *H. atra* of Kanyakumari waters, India was known to have cytotoxicity effect on Human cervix adenocarcinoma cell (HeLa), human breast adenocarcinoma (MCF7), Hep2, and Vero cells.[15,16] Whereas Murniasih *et al*.[17] investigated antioxidant capacity on *H. atra* extract from Lombok waters, Indonesia. To the best of our knowledge, a study to reveal the mechanism of *H. atra* extract inhibition on cancer cell lines through apoptosis induction has not been done before. This study aims to determine the cytotoxicity and induction of apoptosis of *H. atra* ethanol extract against several types of cancer cell lines and to investigate the active compounds contained in the ethanol extract.

MATERIALS AND METHODS

Sampling and identification of sea cucumber

Sea cucumber samples were taken from the waters of Halmahera, North Maluku Province, Indonesia. Samples obtained were cleaned up from its viscera and then were stored in a cool box and immediately were transported to the laboratory. Identification was conducted at the Center for Oceanographic Research, Indonesian Institute of Sciences, Jakarta.

Extraction and fractionation

The sample was washed with fresh water then cut into pieces of approximately 2–4 cm^3. The extraction was conducted by 24 h maceration using 96% ethanol. The extract was then concentrated using a vacuum concentrator. The concentrated extract was then frozen before it was dried using a freeze drier. The dried extracts were further fractionated by gradual partitioning using n-hexane, ethyl acetate, and methanol-water. The latter fraction was then subjected into C18 column to obtain 9 fractions. The active fraction was then injected into liquid chromatography-ion trap-time of flight-mass spectrophotometer (LC-IT-ToF-MS) (Shimadzu) using Luna C18 2.0 mm × 100 mm column, the sample injected volume was 20 µl and, The sample was eluted using water-acetonitrile gradient system for 30 min with the flow rate was settled in 0.2 ml/min. The rest settings were ionization mode electrospray ionization (ESI), probe and needle voltage 4.5 kV, and detector voltage 1.7 kV.

Phytochemical screening

Phytochemical screening was carried out for alkaloids, flavonoids, steroids-triterpenoids, phenols, saponins, and tannins following Siddiqui and Ali[18] with some modifications. These screening were carried out for ethanol extracts of *H. atra*.

Cytotoxicity assay

Cytotoxicity assay was conducted according to Ebada *et al*.[19] with slightly modifications. The cytotoxicity test was performed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method against human breast ductal carcinoma cells (T47D), human breast adenocarcinoma (MFC7), human colorectal adenocarcinoma (WiDr), and human cervix adenocarcinoma (HeLa). All cells were routinely grown and maintained in Roswell park memorial institute medium with 10% fetal bovine serum and 1% penicillin-streptomycin. All cell lines were incubated in a moisture-saturated atmosphere containing 5% CO_2. To each well of the 96-wells microplate containing 100 µL of cell suspension (1.5×10^4 cells) was added 100 µL of extract in the various concentration (2.5; 5; 10; 20 and 40 µg/ml) and the plate was then incubated in a CO_2 incubator at 37°C for 24 h. After the addition of 100 µL of MTT saline solution (0.5 mg/mL) to each well of microplate, the plate was then incubated for 4 h under the same conditions in the CO_2 incubator. The cell morphology and the formed formazan crystal were documented, and then, the absorbance was measured at 570 nm in a microplate reader (Thermo Scientific). The value of Inhibition concentration 50 (IC_{50}) was determined using MINITAB probit analysis version 16.0 (Minitab, Inc).

Fluorescein isothiocyanate Annexin V staining

The analysis and discrimination between apoptosis and necrosis cancer cells was conducted using Fluorescein isothiocyanate (FITC) Annexin-V-Apoptosis Detection Kit II (BD Pharmagen).[20] The test method was conducted using protocols from the kit. In summary, the test methods were the following: after T47D cells were treated with 10 µg/ml of extract for 24 h, the cells were trypsinized and washed with cold Phosphate Buffer Saline (PBS) (two times) then the cells were resuspended with 1X binding buffer at a concentration of 1×10^6 cells/ml. As much as 100 µl solution (1×10^5 cell) were transferred to a 5 ml culture tube then was added with 5 µl of FITC Annexin V and 5 µl propidium iodide (PI). The mix solution gently homogenized using vortex and incubated 15 min at room temperature (25°C) in the dark. After incubation, the mix solution added with 400 µl binding buffer. Apoptotic and necrotic cells were analyzed by flow cytometer (BD Accuri C6).

Active caspase-3 staining

Caspase-3 is an activated cysteine protease in the early apoptosis stadia. Caspase-3 detection of the T47D cell treated with *H. atra* ethanol extract after 24 h was performed by flow cytometry using FITC Active Caspase-3 Apoptosis Kit (BD Pharmingen).[21] The test was performed as per protocol in the kit. The treated T47D cells were washed twice with cold PBS. The cell pellet was resuspended with Cytofix/CytopermTM solution with a concentration of 1×10^6 cells/0.5 ml and was incubated for 20 min on ice. Pellet cells were aspirated, and solution Cytofix/Cytoperm™ was discarded then pellet was washed with BD Perm/Wash™ buffer (1X) at a volume of 0.5 ml buffer/1×10^6 cells at room temperature. The cell was resuspended with BD Perm/Wash™ buffer (1X) plus antibody and incubated for 30 min at 25°C. Subsequently, each test was washed in 1.0 ml BD Perm/Wash™ buffer (1X), then, the test was resuspended in 0.5 ml BD Perm/Wash™ buffer (1X) and was analyzed by flow cytometry. The analysis of apoptotic and non-apoptotic populations for active caspase-3 was carried out by flow cytometry (BD Accuri C6).

RESULTS

The identification of the specimens conducted at the Research Center for Oceanography Indonesia Institute of Sciences showed that the sample was confirmed as *H. atra* [Figure 1a]. A sample of 5 kg (wet weight) of *H. atra* produced 44.9 g crude extract as an orange powder [Figure 1b].

Figure 1: *Holothuria atra* sea cucumber (a) and ethanol extract of sea cucumber *Holothuria atra* (b)

Phytochemical screening results showed that the ethanol extract positively contained alkaloids, flavonoids, steroids-triterpenoids, phenols, and saponins, but negative to tannins.

To evaluate the anticancer potency of *H. atra* ethanol extract, cytotoxicity test was performed using cancer cell line of T47D, MCF7, WiDr, and HeLa. The results showed that the four cells morphologically changed after treated with the ethanol extract for 24 h. Changes in morphology in these four cells were primarily seen at a dose of 10 µg/ml [Figure 2]. This was consistent with a cell mortality profile in which about 40-50% of the treated cells died at the concentration of 10 µg/ml extract. The calculation of IC_{50} value showed that ethanol extract of *H. atra* had strong cytotoxicity against T47D, MCF7, WiDr, and Hela cells with IC_{50} value of 9.6; 14.3; 11.4; and 10.4 µg/ml, respectively [Table 1].

T47D cells were then used to see the effect of apoptotic induction of the ethanol extract. Analysis of apoptotic induction was conducted using flow cytometry with FITC Annexin V as the detection kit. The result showed that the apoptotic cell population treated with the ethanol extract of *H. atra* (dose of 10.0 µg/ml) for 24 h was 73.7%. We used doxorubicin as a positive control (1.0 µg/ml), resulting 65.5% of cells underwent apoptosis. This was, in contrast, 86.5% of the cell population was largely still in a viable (cells without treatment (negative control) [Figure 3].

Table 1: Cell mortality profile and the IC_{50} value of *Holothuria atra* extract against some cancer cell lines

Concentration (µg/ml)	Cell mortality (%)			
	T47D cell	MCF7 cell	WiDr cell	HeLa cell
2.5	0	8.5±7.9	4.1±2.2	14.3±1.1
5	35.8±0.6	38.1±7.5	22.2±0.1	32.5±12.0
10	53.2±4.9	51.5±8.8	46.8±1.1	40.5±2.1
20	77.9±11.8	52.2±4.3	79.4±3.8	72.5±10.4
40	89.8±4.4	67.3±5.7	81.9±1.4	83.9±10.6
IC_{50} value (µg/ml)	9.6	14.3	11.4	10.4

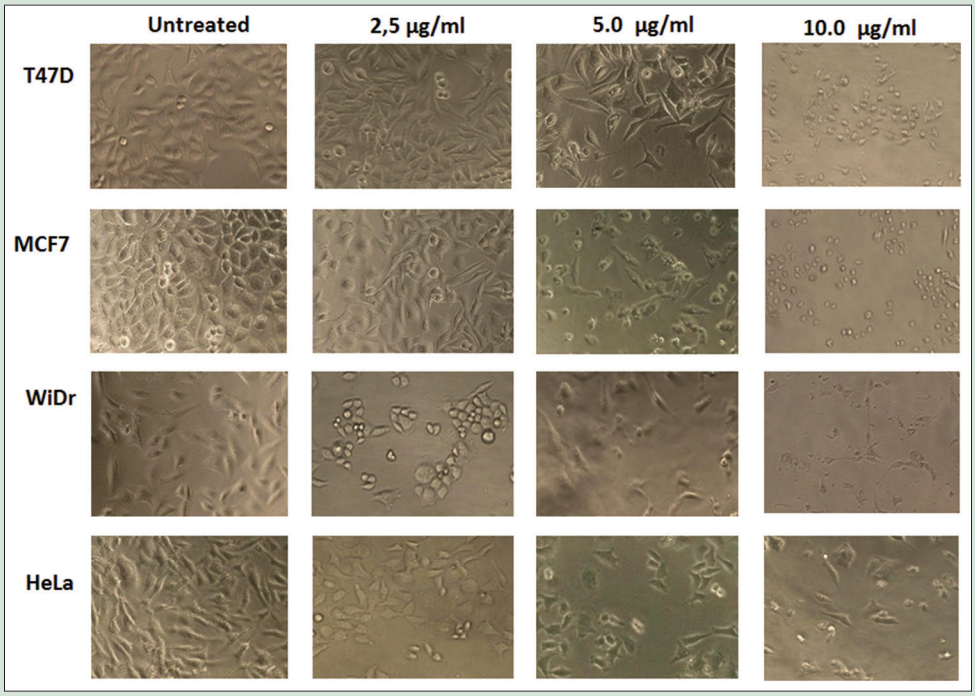


Figure 2: Effect of *Holothuria atra* ethanol extract on cell morphology T47D, MCF7, WiDr, and HeLa for 24 h

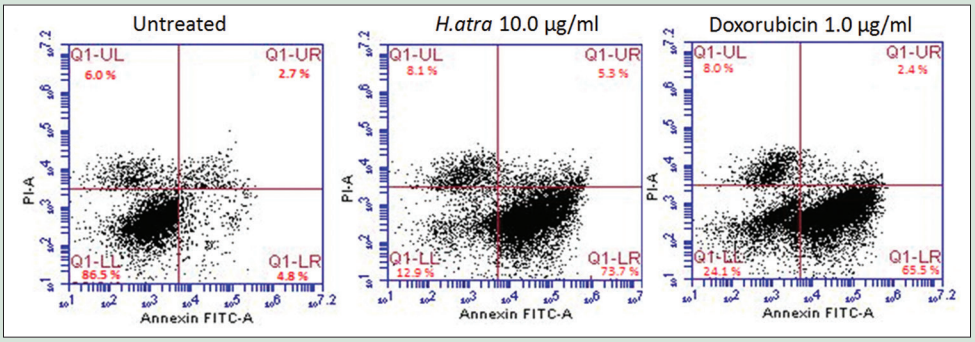


Figure 3: Flow cytometric analysis of Fluorescein isothiocyanate Annexin V staining of T4D7 cell populations after treated with *Holothuria atra* ethanol extract for 24 h compared to untreated and positive control (doxorubicin)

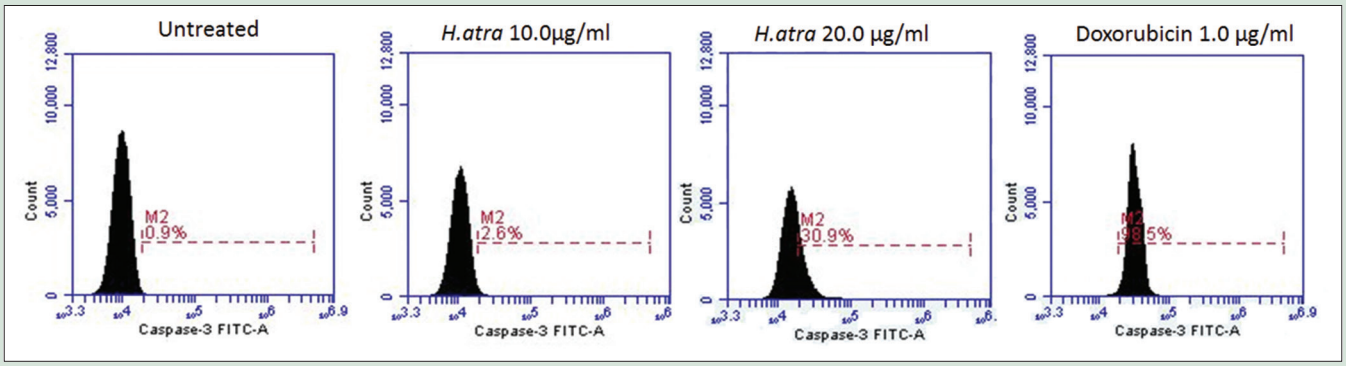


Figure 4: Flow cytometric analysis of apoptotic and non-apoptotic T47D cell populations for active caspase-3 at extracts doses of 10.0 µg/ml and 20.0 µg/ml compared with positive control (doxorubicin) and untreated cells after 24 h incubation

To ensure that the ethanol extract induced apoptosis in T47D cells, we further performed a caspase-3 detection test. Caspase-3 as one of the key proteins in the apoptotic process can be used as a marker on cells undergoing apoptosis in early apoptosis. Results showed the presence of caspase-3 activation, especially at 20.0 µg/ml dose, of 30.9% [Figure 4]. T47D cells treated with doxorubicin (positive control) showed greater caspase-3 activation of 98.5%. This was, in contrast, T47D cells without treatment showed only 0.9% caspase-3 activation.

Identification of compounds in active fraction

The results of the cytotoxicity test on the liquid-liquid partition fraction showed that the methanol-water fraction had the best cytotoxicity (IC_{50} value of 16.5 µg/ml) compared to the *n*-hexane fraction and ethyl acetate fraction [Table 2]. The methanol-water fraction was further fractionated using the C18 flash column to yield 7 fractions. The fraction F1 to F4 had no cytotoxicity effects while fraction F5, F6, and F7 showed cytotoxicity with IC_{50} values of 7.4; 17.2 and 11.9 µg/ml, respectively, against HeLa cells. Based on this, the LC-IT-ToF-MS analysis was performed on fraction F5 to know the possible active compounds that responsible for their cytotoxicity.

Based on the LC-IT-ToF-MS chromatogram, there were two major compounds in fraction F5. Both compounds were eluted at the min of 13.86 and 16.75 [Figure 5a]. The first compound had a major molecular ion peak (*m/z*) of 763.2197 $[M + H]^+$. The peak ion with the value of *m/z* 763.2197 ($C_{41}H_{62}O_{13}$) was most likely a triterpene compound attached to a glycoside. This was supported by the detection of molecular ions at *m/z* 485.4257 $[M + H]^+$ which was the molecular ions peak of an aglycone compound called Cucumechinol [Figure 5b] ($C_{30}H_{44}O_5$) which had lost its deoxyhexose glycoside group. The second compound with a retention time of 17.58 has a major molecular ion peak with *m/z* value of 471.3838 $[M + H]^+$. The molecular ion peak is identical to an aglycone called Philinopgenin B [Figure 5c]. The structure of Cucumechinol and Philinopgenin B compounds according to Miyamoto *et al.*[22] and Zhang *et al.*[23] are shown in Figure 6.

Table 2: Results of fractionation of *Holothuria atra* crude extract and IC_{50} values of cytotoxicity test

Extract and fractions	Yield	IC_{50} value (µg/ml) to HeLa cell
Crude extract	20.0 g	10.4
n-Hexane fraction	3.9 g	334.0
Ethyl acetate fraction	6.0 g	95.9
Methanol-water fraction	10.0 g	16.4
F1–F4 fractions	0.121-2334.0 g	>500 (no active)
F5 fraction	1.4 g	7.4
F6 fraction	246.1 mg	17.2
F7 fraction	112.6 mg	11.9

DISCUSSION

Sea cucumber belonging to the class *Holothuroidea* phylum Echinodermata, are echinoderms that are phylogenetically related to sea stars, sea urchins and sea lilies.[24] Sea cucumbers are benthic invertebrate which contains many active compounds. This active compound is secreted as part of its defense mechanism against predator attack. One of the many active compounds contained in the body of sea cucumbers is a saponin (triterpene glycosides). The result of the phytochemical test showed that the crude extract of *H. atra* positively contained saponin.

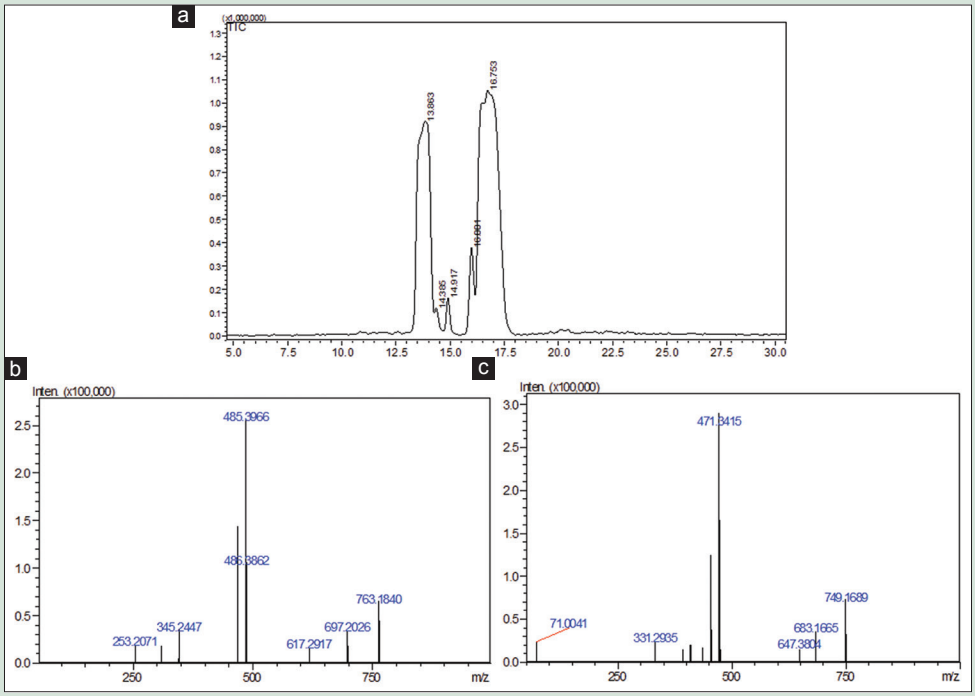


Figure 5: LC-IT-ToF-MS active fraction analysis of F5, mass spectrometry chromatogram (a), molecular ion of compound 1 (b) and molecular ion of compound 2 (c)

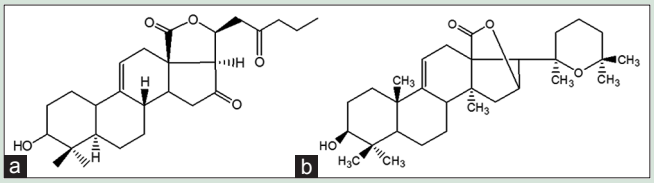


Figure 6: Structure of Cucumechinol (a) and Philipnogenin B (b)

By performing the LC-IT-ToF-MS analysis of the active fraction, Cucumichenol and Philinopgenin B were detected. Both of them belong to the triterpene glycoside. Saponin on many sea cucumbers is contained within its body walls.[25] So far, about 150 types of saponins are found in sea cucumber *Holothuroidea*.[26]

Structurally, the holothurian saponin is a triterpene glycoside composed of an oligosaccharide chain and an aglycone which is predominantly by a holostane type. However, recent investigation results also showed that some triterpenes do not contain holostane aglycone structure (nonholostane aglycone structure). In the structure of the oligosaccharide, the first monosaccharide unit is always a xylose, while 3-O-methylglucose dan 3-O-methylxylose is always at terminal. In some glycosides, sulfate groups are attached to the oligosccaharide chain. Most of them are mono-sulfated glycosides with few occurrences of di- and tri-sulafted glycosides.[27,28]

The results of this study showed that *H. atra* ethanol extract had good cytotoxicity against T47D, MCF7, WiDr, and HeLa cells with IC_{50} values ranging from 9.6-14.3 μg/ml. The active fraction (F5) had the best cytotoxicity with an IC_{50} value of 7.4 μg/ml. Other research results showed that *H. atra* from Mentawai islands, West Sumatera, Indonesia, had cytotoxicity against Supris clone-1 cell (SP-C1).[29] Dhinakaran and Lipton[16] showed that methanolic extract of the *H. atra* from Southeast Coast of India had cytotoxicity against Hep2 cells.

The cytotoxicity of *H. atra* ethanol extract against T47D cells occurred through induction of apoptosis. This was detected by flow cytometry analysis. Through this analysis, the population of apoptotic, necrosis, and viable cells can be clearly distinguished. After exposure to *H. atra* extract for 24 h at a dose of 10 μg/ml, 73.7% of T47D cell population underwent apoptosis [Figure 3]. The apoptotic induction capability of the extract against T47D cells was confirmed by caspase-3 activation.

One of the changes that occur in apoptotic cells is the translocation of phosphatidylserine (PS) from the inner part to the outer layer of the plasma membrane. PS exposure on the outside of the plasma membrane can be detected using Annexin V.[30,31] Annexin V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes including FITC. This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation.[20] PS exposure also occurs in necrotic cells. To distinguish between apoptotic and necrotic cells, in general, Annexin V is used together with a PI. PI will mark necrotic cells, but not apoptotic cells. In this assay, Annexin V binds the phospholipid PS, marking apoptotic and necrotic cells, while PI binds DNA, marking only necrotic cells.[32]

Caspase-3 is a main executioner of apoptotic cell death. Caspase-3 (together with caspase-6 and -7) is classified as executioner caspases, with the main function in the late events of apoptotic cell death.[33] Caspase-3 is the most widely studied of the effector caspases. It plays a key role in both the death receptor pathway, initiated by caspase-8, and the mitochondrial pathway, involving caspase-9. Several studies have shown that caspase-3 activation was needed to induce apoptosis in response to chemotherapy drugs such as doxorubicin. Caspase-3 is produced as an inactive 32-kDa proenzyme, which is cleaved at an aspartate residue to yield a 12-kDa and a 17-kDa subunit. Two 12-kDa and two 17-kDa subunits combine to form the active caspase-3 enzymes. Caspase-3 cleaves a wide range of cellular substrates including structural

proteins (e.g., lamins) and DNA repair enzymes (e.g., poly-[ADP-ribose] polymerase). It also activates an endonuclease caspase-activated DNAse, which causes the DNA fragmentation that is the characteristic of apoptosis.[34] In this study, caspase-3 in T47D cells treated with *H. atra* ethanol extract was detected through flow cytometry analysis. A total of 30.9% T47D cells activated caspase-3. The activation of caspase-3 induced by doxorubicin as a positive control reached 98.5%) [Figure 4]. Our study results have confirmed several previous studies that explained triterpene glycoside derived from sea cucumber-induced apoptosis on cancer cell line through the activation of intracellular caspase cell death pathways including cell cycle arrest of at S or G2/M phases.[24]

In this study, the cytotoxic effect of *H. atra* was related to the triterpene glycoside in the extract. Several anticancer compounds of triterpene glycoside from sea cucumbers that have anticancer effects through apoptosis induction are Philinopside A (from *Pentacta quadrangularis*), Holothurin A (from *Pearsonothuria graeffei*), and Frondoside A (from *Cucumaria frondosa*).[24] The cytotoxicity and apoptosis induction of *H. atra* ethanol extract have been proven although further research is needed to elucidate the structure of the active chemical compound and to understand the molecular mechanism of the active compound in inhibiting the proliferation of cancer cells.

CONCLUSIONS

Ethanol extract of *H. atra* had cytotoxicity against T47D, MCF7, WiDr, and HeLa cells with IC_{50} value of 9.6, 14.3, 11.4, and 10.4 µg/ml, respectively. The ethanol extract induced apoptosis and activated caspase-3 in T47D cells. The compounds responsible for the bioactivity were triterpene glycosides which are most likely Cucumechinol and Philinopgenin B.

Acknowledgment

The author would like to thank Sri Iswani for providing MS data, also to Erni Halimatushadyah and Nurul Mutia Putram for helping during experiment.

Financial support and sponsorship

This research was supported by the Indonesia Research Center for Marine and Fisheries Product Processing and Biotechnology, Ministry of Marine Affairs and Fisheries, Republic of Indonesia.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. American Cancer Society. Cancer Facts and Figures 2017. Atlanta: American Cancer Society; 2017. Available from: https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/annual-cancer-facts-and-figures/2017/cancer-facts-and-figures-2017.pdf. [Last accessed on 2018 Apr 04].
2. Ferreira CG, Epping M, Kruyt FA, Giaccone G. Apoptosis: Target of cancer therapy. Clin Cancer Res 2002;8:2024-34.
3. Genderen HV, Kenis H, Dumont E, Heerde WV, Hofstra L, Reutelingsperger CP. Membrane alteration in dying cells. In: Hughes D, Mehmet H, editors. Cell proliferation and Apoptosis. Oxford: BIOS Scientific Publisher Limited; 2003. p. 185-6.
4. Ghobrial IM, Witzig TE, Adjei AA. Targeting apoptosis pathways in cancer therapy. CA Cancer J Clin 2005;55:178-94.
5. Matsushita H, Kuwabara H, Ishikawa S, Mochizuki M. Apoptosis induced in human cell lines by a butanol extract from *Chlorophytum comasum* roots. J Health Sci 2005;51:241-345.
6. Koff JL, Ramachandiran S, Bernal-Mizrachi L. A time to kill: Targeting apoptosis in cancer. Int J Mol Sci 2015;16:2942-55.
7. Ruiz-Torres V, Encinar JA, Herranz-López M, Pérez-Sánchez A, Galiano V, Barrajón-Catalán E, *et al.* An updated review on marine anticancer compounds: The use of virtual screening for the discovery of small-molecule cancer drugs. Molecules 2017;22. pii: E1037.
8. Imperatore C, Aiello A, D'Aniello F, Senese M, Menna M. Alkaloids from marine invertebrates as important leads for anticancer drugs discovery and development. Molecules 2014;19:20391-423.
9. Zhang J, Zhang H, Alexandre Muehlmann L, Jiang CS, Guo YW. Synthesis and structural modification of marine natural products. Molecules 2017;22. pii: E882.
10. Purcell SW. Value, market preferences and trade of beche-de-mer from pacific island sea cucumbers. PLoS One 2014;9:e95075.
11. Setyastuti A, Purwati P. Species list of Indonesian trepang. SPC Beche Mer Inf bull 2015;35:19-25.
12. Janakiram NB, Mohammed A, Rao CV. Sea cucumbers metabolites as potent anti-cancer agents. Mar Drugs 2015;13:2909-23.
13. Bordbar S, Anwar F, Saari N. High-value components and bioactives from sea cucumbers for functional foods – a review. Mar Drugs 2011;9:1761-805.
14. Bahrami Y, Zhang W, Chataway T, Franco C. Structure elucidation of five novel isomeric saponins from the viscera of the sea cucumber *Holothuria lessoni*. Mar Drugs 2014;12:4439-73.
15. Dhinakaran DI, Lipton AP. Bioactive compounds from *Holothuria atra* of Indian Ocean. Springerplus 2014;3:673.
16. Dhinakaran DI, Lipton AP. Antitumor and antifungal activities of organic extracts of sea cucumber *Holothuria atra* from the Southeast Coast of India. J Ocean Univ China 2015;14:185-9.
17. Murniasih T, Putra MY, Pangestuti R. Antioxidant capacities of *Holothuria* sea cucumbers. Ann Bogorienses 2015;19:21-6.
18. Siddiqui AA, Ali M. Practical Pharmaceutical Chemistry. 1st ed. New Delhi: CBS Publishers and Distributors; 1997.
19. Ebada SS, Edrada RA, Lin W, Proksch P. Methods for isolation, purification and structural elucidation of bioactive secondary metabolites from marine invertebrates. Nat Protoc 2008;3:1820-31.
20. Technical Data Sheet. FITC Annexin V Apoptosis Detection Kit I. BD Pharmingen™. 556547 Rev. 5. New Jersey: Becton, Dickinson and Company; 2008.
21. Technical Data Sheet. PE Active Caspase-3 Apoptosis Kit. BD Pharmingen™. 550914 Rev. 5. New Jersey: Becton, Dickinson and Company; 2015.
22. Miyamoto T, Togawa K, Higuchi R, Komori T. Constituents of *Holothuroidea*, I. Isolation and structures of three triterpenoid aglycones, cucumechinol A, B, and C, from the sea cucumber *Cucumaria echinata*. Liebigs Ann Chem 1990;1:39-42.
23. Zhang SH, Li L, Yi YH, Zou ZR, Sun P. Philinopgenin A, B, and C, three new triterpenoid aglycones from the sea cucumber *Pentacta quadrangulasis*. Mar Drugs 2004;2:185-19.
24. Aminin DL, Menchinskaya ES, Pisliagin EA, Silchenko AS, Avilov SA, Kalinin VI, *et al.* Anticancer activity of sea cucumber triterpene glycosides. Mar Drugs 2015;13:1202-23.
25. Van Dyck S, Gerbaux P, Flammang P. Qualitative and quantitative saponin contents in five sea cucumbers from the Indian Ocean. Mar Drugs 2010;8:173-89.
26. Honey-Escandón M, Arreguín-Espinosa R, Solís-Marín FA, Samyn Y. Biological and taxonomic perspective of triterpenoid glycosides of sea cucumbers of the family *Holothuriidae* (*Echinodermata*, *Holothuroidea*). Comp Biochem Physiol B Biochem Mol Biol 2015;180:16-39.
27. Caulier G, Dyck SV, Gerbaux P, Eeckhaut I, Flammang F. Review of saponin diversity in sea cucumbers belonging to the family *Holothuriidae*. SPC Beche Mer Inf Bull 2011;31:48-54.
28. Kim SK, Himaya SW. Triterpene glycosides from sea cucumbers and their biological activities. Adv Food Nutr Res 2012;65:297-319.
29. Satari MH, Arma U, Ilyas S, Handayani D. Anticancer potency of black sea cucumber (*Holothuria atra*) from Mentawai Islands, Indonesia. Padjadjaran J Dent 2017;29:54-7.
30. Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: Mechanistic description of dead and dying eukaryotic cells. Infect Immun 2005;73:1907-16.
31. Elmore S. Apoptosis: A review of programmed cell death. Toxicol Pathol 2007;35:495-516.
32. Ranalli M, Oberst A, Corazzari M, Laurenzi VD. Flow cytometric studies of cell death. In: Hughes D, Mehmet H, editors. Cell Proliferation and Apoptosis. Oxford: BIOS Scientific Publisher Limited; 2003. p. 338-40.
33. Garcia-Faroldi G, Melo FR, Rönnberg E, Grujic M, Pejler G. Active caspase-3 is stored within secretory compartments of viable mast cells. J Immunol 2013;191:1445-52.
34. O'Donovan N, Crown J, Stunell H, Hill AD, McDermott EM, O'Higgins N, *et al.* Caspase 3 in breast cancer. Clin Cancer Res 2003;9:738-42.