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Comparison of metabolite profiles and cytotoxicity of the black sea cucumber (*Holothuria atra*) dried with different drying techniques

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Abstract

Holothuria atra is a species of sea cucumber that could be a source of cytotoxic compounds. The present work aimed to determine the effect of different drying techniques on the profile of metabolites and cytotoxicity of sea cucumber extracts. Sea cucumbers were dried in the sun for 3 d, and oven-dried at a temperature of 40, 50, and 60°C for 24 h. Fresh sea cucumbers without drying served as a control. The dried and fresh sea cucumbers were extracted by maceration for 12 h using ethanol. The metabolite profiling was performed using Fourier-transform infrared spectroscopy (FTIR) and high-performance liquid chromatography (HPLC) analyses, whereas the cytotoxicity was assayed by MTT method using the human breast ductal carcinoma cells (T47D). Analyses of FTIR and HPLC showed that the bioactive compounds of the ethanol extract were relatively stable during drying. This was in line with its cytotoxicity against T47D cells, which did not show significant differences between treatments. Based on these results, it was concluded that drying by sun or oven did not damage the bioactive compounds in the ethanolic extract of sea cucumber.

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Introduction

Secondary metabolites play an important role as a starting point in the drug discovery process (Mudianta *et al.*, 2016). They are also a source of medicinal compounds that have diverse chemical structures with a wide range of bioactivities (Aminin *et al.*, 2015). Anticancer compounds are one of the targets for marine drug discovery since cancer is one of the major causes of death globally (American Cancer Society, 2017). Research on the pharmacological activities of the marine environment conducted by the U.S. National Cancer Institute over a period of 15 years found that approximately 4% of the marine species examined had anticancer activity (Janakiram *et al.*, 2015).

Sea cucumbers (phylum Echinodermata, class Holothuroidea) have long been used by the Asians as foods and traditional medicines. They contain high-quality nutrients such as proteins, amino acids, vitamins, minerals, and fatty acids. In addition, they also contain various bioactive metabolites such as saponins, chondroitin sulphate, glycosaminoglycans,

sulphated polysaccharides, sterols, peptides, cerebrosides, unsaturated fatty acids, and lectins. Their bioactivities include as an immunostimulant, antiangiogenic, anticancer, anticoagulant, anti-hypertension, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, and wound healing (Bordbar *et al.*, 2011; Khotimchenko, 2018).

The most prominent bioactive compound as an anticancer in sea cucumbers is saponin. Saponins in many sea cucumbers are found in the body wall and viscera (Dyck *et al.*, 2010). So far, around 150 types of saponins have been identified from sea cucumbers of class Holothuroidea (Honey-Escandón *et al.*, 2015). Results of previous studies reported that the sea cucumber *Holothuria atra* had promising cytotoxicity against several cancer cell lines, namely human breast ductal carcinoma (T47D), human cervix epitheloid carcinoma (HeLa), human colon adenocarcinoma (WiDr), human Caucasian hepatocyte carcinoma (HepG2), and human oral tongue squamous cell carcinoma (SP-C1) (Satari *et al.*, 2017; Halimatushadyah *et al.*, 2018). Furthermore, the ethanolic extract of *H. atra* showed

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induction of apoptosis and caspase-3 activation in T47D breast cancer cells. The bioactive metabolites most likely responsible for its activity were saponins, which include echinoside B, philinopgenin, and cucumichenol (Nursid *et al.*, 2019). Echinoside B that was purified from *H. atra* had cytotoxicity against human Caucasian lung carcinoma (A549) and mouse epithelial melanoma (B16F10) cells, with an IC₅₀ value of 0.5 - 2.5 µM. Molecular docking simulation showed that echinoside B was bound very strong with the catalytic domain of PAK1 (p21-261 activated kinase 1) (Shahinozzaman *et al.*, 2018). These studies revealed that *H. atra* could be a source of cytotoxic saponin compounds that can be developed as anticancer agents.

In developing bioactive compounds from nature as medicinal materials, the problem lies in the drying process of the raw materials. This is related to the stability of the bioactive compounds in the extract. Ram *et al.* (2017) stated that sea cucumbers are usually processed into a dried product called beche-de-mer. For industrial-scale production, dry raw materials are easier to handle than wet raw materials. In addition, the cost of transportation of dry materials is also cheaper than wet materials. However, during drying, the amount of bioactive compounds is often diminished, accompanied by a decrease in its bioactivity. Therefore, the aim of the present work was to determine the metabolite profiles and cytotoxicity of sun- and oven-dried *H. atra* ethanolic extract as compared to the fresh extracts.

Materials and methods

Sample collection

Sea cucumbers (*H. atra*) were obtained from the waters around Jailolo Island, North Maluku, Indonesia. Their stomach content was then immediately removed, and the cleaned sea cucumbers were preserved at cold temperatures (6 - 10°C) in a cool box. Subsequently, the samples were brought to the laboratory, and stored at -20°C for future analyses.

Sample preparation and drying process

The weight of each sea cucumber was 20 - 40 g with a length of 8 - 12 cm. Sea cucumbers were cut into small pieces ($\pm 2 - 3 \text{ cm}^3$), and then boiled for 30 min to remove salt residues, prevent odours, and prevent spoilage during drying. Sea cucumbers were dried using two techniques: sun-drying (PM) and

oven-drying (PO). For sun-drying, sea cucumbers were dried under the sun for 3 d (starting at 8 am to 5 pm), and for oven-drying, sea cucumbers were dried in an oven at 40°C (POA), 50°C (POB), and 60°C (POC) for 24 h. For control, fresh sea cucumber (TS) extracts that did not undergo boiling and drying was used.

Solvent extraction

Extraction was performed by the maceration method using ethanol. Sea cucumbers were ground to powder prior to maceration. This was done in 12 h for three times, at a ratio of 1:2 (w/v). The extract was filtered using Whatman filter paper 41 with pore size of 20 µm. The filtrate was then evaporated using a vacuum rotary evaporator. The remaining ethanol in the extract was dried using a freeze dryer until the optimum crude extract was obtained.

Proximate composition analysis

Analyses of ash, protein, lipid, and total carbohydrate contents were performed following the Association of Official Analytical Chemists (AOAC) methods (AOAC, 2016). The ash content was determined by incineration at 550°C. The protein content ($\text{N} \times 6.25$) was determined by the Kjeldahl method. The lipid content was determined by the Soxhlet extraction method. The total carbohydrate content was determined by difference.

Cytotoxicity test

Cytotoxicity test was performed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT method). The assay procedure was carried out following Mosmann (1983) as described by Ebada *et al.* (2008). The human breast ductal carcinoma cells (T47D) were used in cytotoxicity evaluation. Cells were maintained in RPMI media supplemented with 10% FBS, 2% penicillin-streptomycin, and 0.5% fungizone. Cells were also cultured in CO₂ incubator at 37°C, with the CO₂ flow of 5 mL/min. The extract dose used for the assay was 30 µg/mL.

FT-IR analysis

Extracts for FT-IR analysis were carried out in the form of potassium bromide (KBr) pellets. About 1 - 2 mg of extract was mixed with 100 mg of KBr. The mixture was then ground using mortar. Samples were then analysed by FT-IR (Perkin Elmer).

Active fraction analysis

The analysis of active fractions used as reference was performed using assay guided fractionation. The assay used in selecting the active fraction was the cytotoxicity test. The active fraction separation method was carried out according to Nursid *et al.* (2019). The crude extract was fractionated using *n*-hexane, ethyl acetate, and methanol-water mixture (8:1, v/v). The active fraction was then separated using a C₁₈ column, and eluted using a water-methanol mixture (gradient). The selected active fraction was analysed by HPLC to determine the fingerprint of the active fraction expressed in the HPLC chromatogram. The detection of the presence of bioactive compounds in the extracts was carried out based on the same retention time.

HPLC

The extract and active fraction were dissolved in methanol (HPLC grade), then filtered with a 0.45 µm filter before being injected into the HPLC system. The concentration of the injected extract into the system was 2.5 mg/mL. The HPLC system used

Shimadzu HPLC photodiode array detector (PDA), Phenomenex C₁₈ 100 × 2.0 mm columns, a flow rate of 0.2 mL/min, and an elution system starting with 15% acetonitrile in H₂O to 100% acetonitrile concentrations for 30 min, followed by elution of 100% acetonitrile for 20 min.

Statistical analysis

The data obtained from three replications were analysed using a One-way analysis of variance (ANOVA) followed by a *post-hoc* test (Tukey test). Statistical analysis was performed using MINITAB (Version 16.0) to compare the proximate composition and cytotoxicity effect of the extracts. A significance level of 95% ($p < 0.05$) was used throughout the analysis.

Results

Proximate composition of sea cucumber

The proximate composition of sea cucumber dried with different drying techniques is shown in Table 1.

Table 1. Proximate composition of fresh, sun-dried, and oven-dried sea cucumber *H. atra* based on dry weight (DW).

Treatment	Ash (% DW)	Protein (% DW)	Lipid (% DW)	Carbohydrate (% DW)	Moisture (% DW)
Fresh	19.0 ± 0.17 ^a	66.5 ± 3.63 ^b	3.2 ± 0.32 ^a	10.1 ± 3.93 ^a	87.5 ± 0.06 ^a
Sun-dried	8.8 ± 0.75 ^b	78.6 ± 0.89 ^a	2.7 ± 0.26 ^{ab}	9.7 ± 0.50 ^a	7.5 ± 0.14 ^b
Oven-dried (40°C)	8.8 ± 1.21 ^b	78.8 ± 3.90 ^a	2.6 ± 0.41 ^{ab}	9.1 ± 2.08 ^a	7.2 ± 0.43 ^b
Oven-dried (50°C)	8.5 ± 1.22 ^b	80.6 ± 2.21 ^a	2.4 ± 0.25 ^b	8.4 ± 1.01 ^a	8.4 ± 0.61 ^b
Oven-dried (60°C)	7.3 ± 0.80 ^b	82.9 ± 1.52 ^a	2.4 ± 0.11 ^b	6.9 ± 1.51 ^a	4.5 ± 0.92 ^c

Different lowercase superscripts in the same column indicate statistical differences among the treatment.

Different drying techniques did not significantly affect the proximate composition of sea cucumber except for the moisture content. However, when compared to fresh sea cucumbers, the proximate composition of dried sea cucumber was significantly different. The highest ash content was found in fresh sea cucumbers at 19.0%. This was significantly different as compared to dried sea cucumber ($p < 0.05$). However, there was no difference between the ash contents of sun- and oven-dried sea cucumbers. The protein contents of the dried and fresh sea cucumbers varied between 66.5 and

82.9%. The highest value of the protein content was obtained from sea cucumbers that were oven-dried at 60°C. The highest lipid content was found in fresh sea cucumber, which was 3.2% ($p < 0.05$); but this was not significantly different between the drying techniques. The carbohydrate content ranged from 6.9 to 10.1 without any significant differences ($p > 0.05$).

Stability of cytotoxicity

The morphological alteration of T47D cells treated with the sea cucumber extracts obtained from

different drying techniques is shown in Figure 1. For untreated cells, they maintained their original morphology. Untreated cells were also seen to be attached to the surface of the flask, and clearly shaped like epithelial cells. Meanwhile, cells treated with extracts appeared to be degraded and unattached from

the surface of the flask. Evidently showing shrinkage and membrane blebbing, the cells were not sticky, and had irregular shapes. The mortality of T47D cells did not differ significantly between the sun-dried, oven-dried, and fresh sea cucumber extracts ($p > 0.05$) (Figure 2).

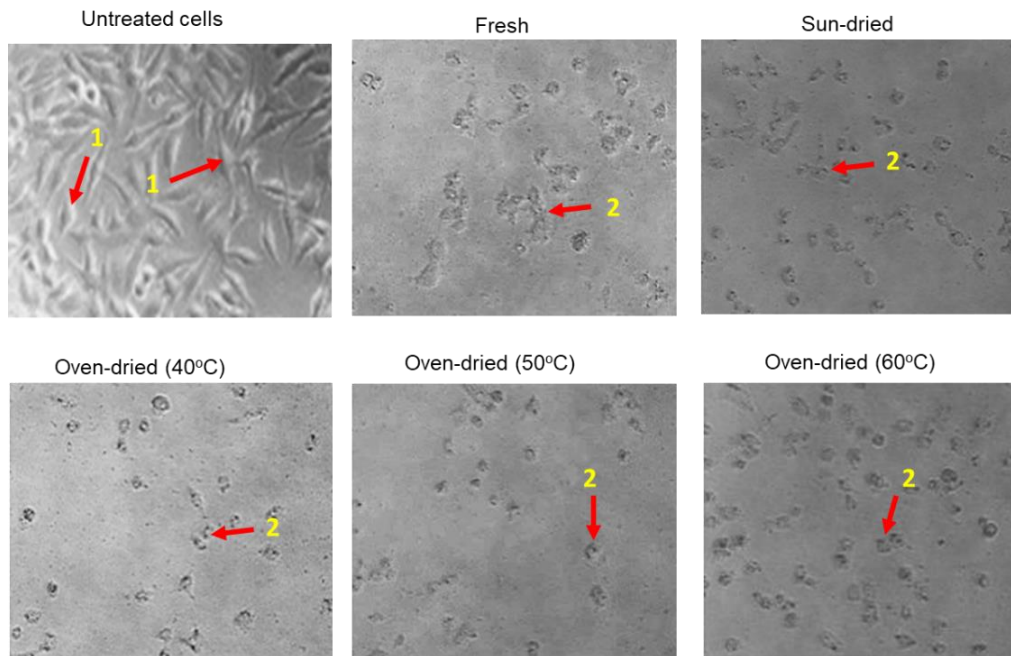


Figure 1. Photomicrographs showing morphological changes of T47D cancer cells after being treated for 24 h at 30 µg/mL of fresh, sun-dried, and oven-dried sea cucumber ethanolic extracts (magnification 40×). Arrows indicate (1) control cells (untreated cells), and (2) treated cells which show degradation or damage (cell shrinkage, membrane blebbing).

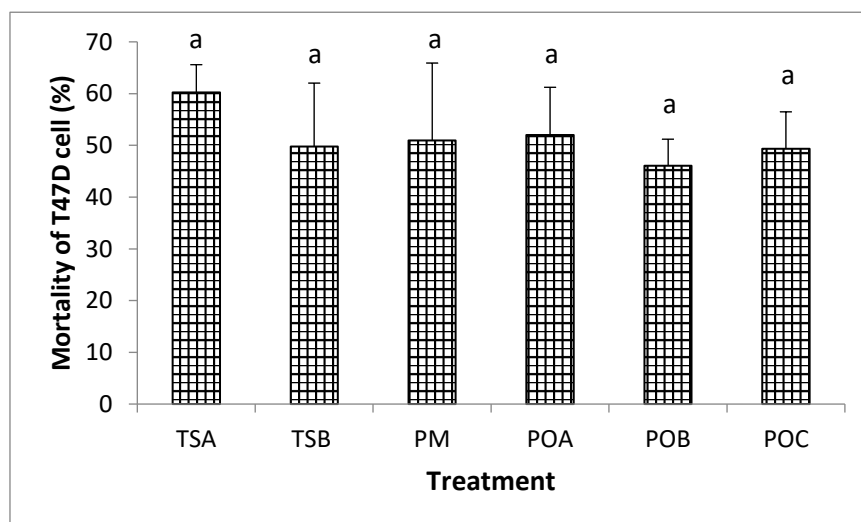


Figure 2. Mortality of T47D cells after being treated with fresh, sun-dried, and oven-dried sea cucumber ethanolic extracts. TSA: fresh without boiling, TSB: fresh with boiling, PM: sun-dried, POA: oven-dried at 40°C, POB: oven-dried at 50°C, and POC: oven-dried at 60°C. Similar lowercase letter indicates that there was no significant difference between the treatments.

FTIR profiles

Metabolite profile analysis of sea cucumber extracts obtained from different drying techniques was performed to see whether there were changes in the functional groups of bioactive compounds in each extract. The FTIR spectra results are presented in Figure 3. FTIR analysis was still very rough to see the metabolite profile in fresh and dried sea cucumbers. Thus, there was a limitation in determining specific saponins through this analysis. Most terpene groups

were in the form of C-C, C=C, and C-H, while many sugars contained C-C and C-O or C=O bonds. These bonds were found in a wide range of vibrations from 3500 to 1054 cm^{-1} . Vibration at around 3390 (O-H^s), 2920 (C-H^s), 1730 (C=O^s), 1630 (C=C^s), and 1090 (C-O^s) at least showed the presence of saponin compounds in the extract. In general, the FTIR profile of each extract had no differences as compared to fresh sea cucumber.

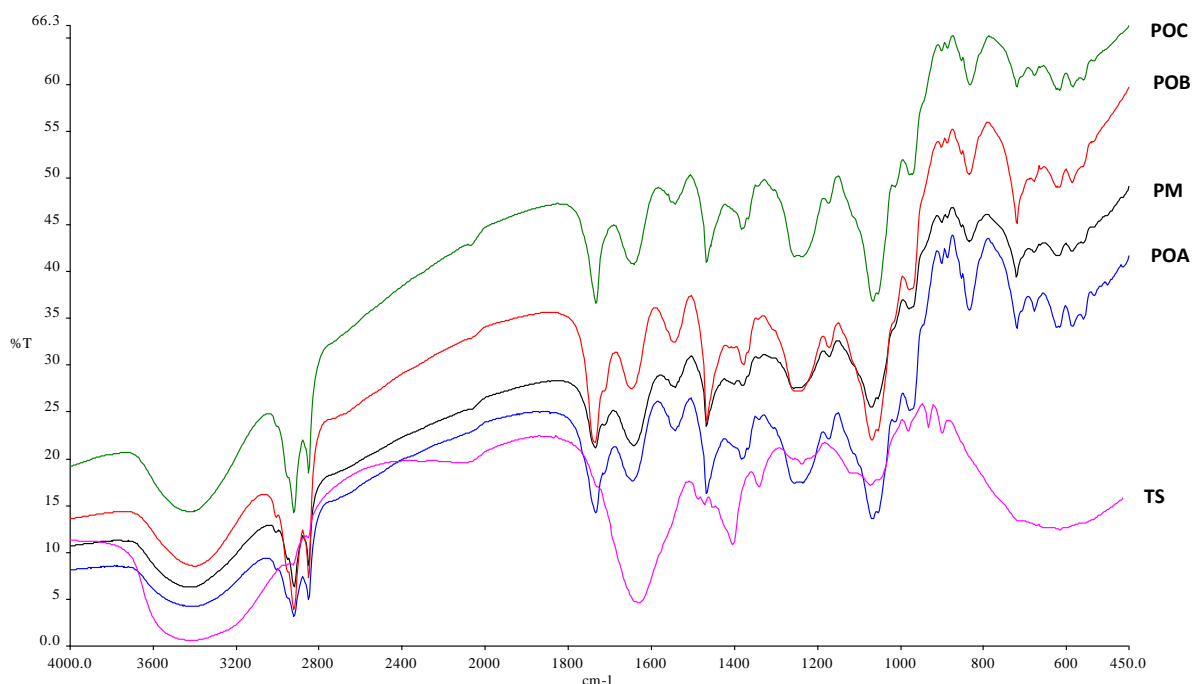


Figure 3. FTIR spectra analysis of fresh, sun-dried, and oven-dried sea cucumber ethanolic extracts. TS: fresh, PM: sun-dried, POA: oven-dried at 40°C, POB: oven-dried at 50°C, and POC: oven-dried at 60°C.

HPLC profiles

Crude extract of sea cucumber had recorded an IC_{50} value of 17.9 $\mu\text{g/mL}$ in the cell. The crude extract partition yielded three fractions namely *n*-hexane, ethyl acetate, and MeOH-water fractions. The IC_{50} values of *n*-hexane, ethyl acetate, and methanol water fractions were 58.5, 54.9, and 14.3 $\mu\text{g/mL}$, respectively. The fractionation of MeOH-water fraction yielded seven fractions. Among them, fraction no. 5 (F5) had the strongest cytotoxicity with an IC_{50} value of 2.8 $\mu\text{g/mL}$. The HPLC chromatograms showed that there were two major peaks in the active fraction (F5) detected at 18.826 and 23.080 min (Figure 4). Since F5 was the most active ($\text{IC}_{50} = 2.8 \mu\text{g/mL}$), the HPLC of this fraction was then used as a reference to detect the presence of bioactive compounds in extracts with various

treatments. Peaks with the same retention time as the major peak at F5 were found in fresh (TS), sun-dried (PM), and 60°C oven dried (POC) extracts. The presence of peaks with the same retention time indicated that the bioactive compounds present in the extract were stable.

Discussion

In the present work, sun-drying and 40 - 60°C oven-drying did not affect the proximate compositions of *H. atra*. This finding is similar with the effect of drying processes on *H. froskali*, in which the protein content was significantly different only after being oven dried at 70°C; however, at temperature less than 70°C, the differences was not significant (Telahigue *et al.*, 2014).

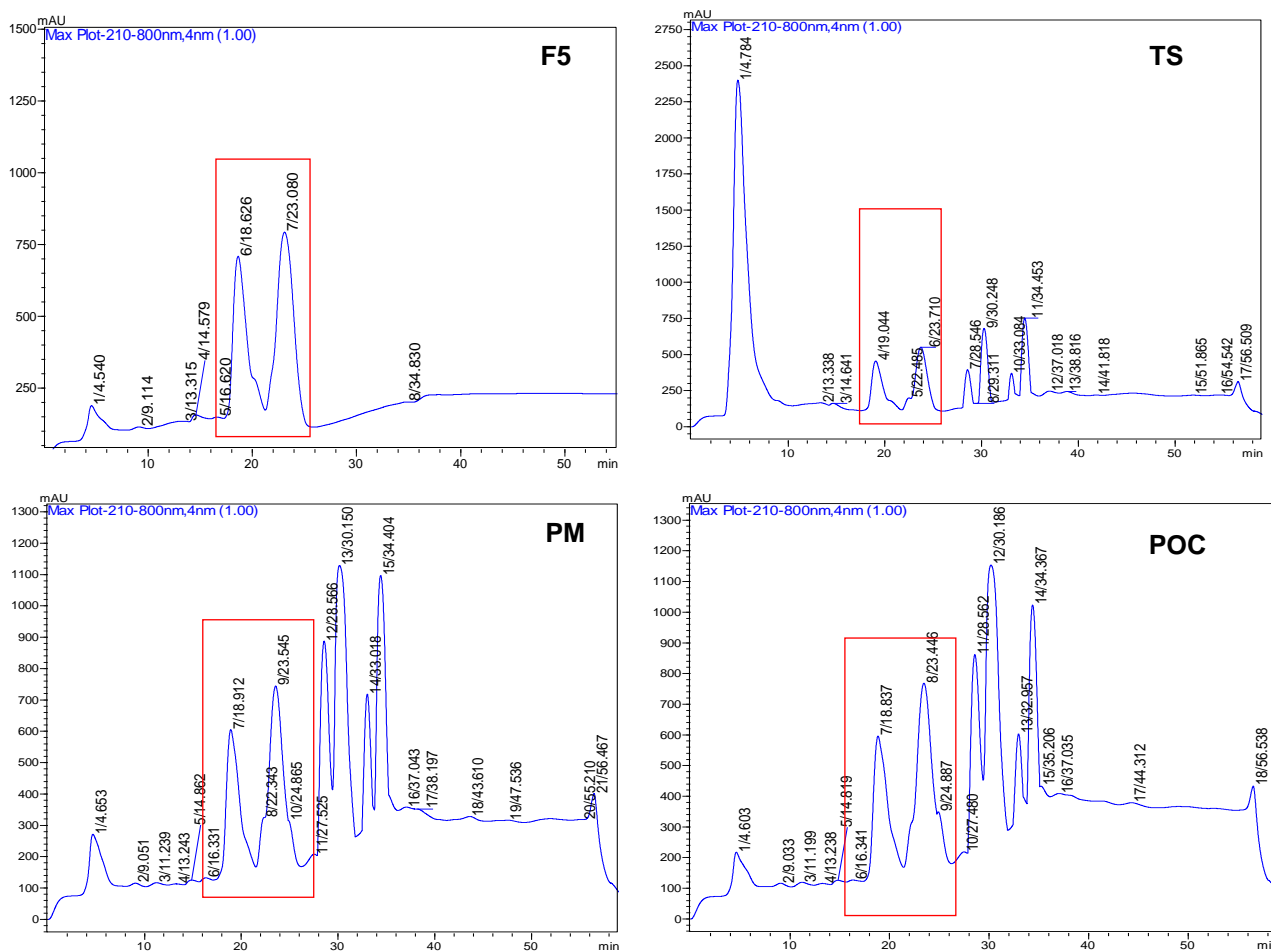


Figure 4. HPLC chromatograms of sea cucumber ethanolic extracts dried through different techniques as compared to active fraction. F5: active fraction as a reference, TS: fresh, PM: sun-dried, and POC: oven-dried at 60°C. The peaks in the red box indicate the active compound in the sea cucumber ethanolic extract.

The total ash content of *H. atra* in the present work ranged from 7.3 to 19.0%. Ibrahim *et al.* (2015) reported 39.5% of *H. atra* ash content. The ash content in *H. tubulosa*, *H. polii*, and *H. mammata* ranged from 5.13 - 7.85% (Aydin *et al.*, 2011). The ash content can be interpreted as a mineral component in a material; the higher the ash content of particular sample, the higher the mineral content of the sample (Nielsen, 2010).

In the present work, *H. atra* yielded high protein content. This is in line with Sibero *et al.* (2019) who found that the protein content of *H. atra* was 83.9%, followed by carbohydrates (10.79%), ash (2.44%), and lipid (1.29%). The high protein content was also found in *H. tubulosa* (66.4%; Cakli *et al.*, 2004) and *H. scabra* (61.94%; Karnila *et al.*, 2011). Sea cucumbers are animals that contain high protein like other fishery products. The proteins in sea

cucumbers have complete amino acids, both essential and non-essential (Chen *et al.*, 2010).

The lipid content of *H. atra* in all treatments ranged from 2.4 to 3.2%. Ibrahim *et al.* (2015) found that the lipid content in *H. atra* was 1.3%. During drying, the lipid content decreases due to the oxidation and auto-oxidation processes. The presence of oxygen acts as a trigger for oxidation reactions, and high storage temperatures can initiate an autoxidation reaction (Kusnandar, 2010).

In the present work, the moisture content of fresh *H. atra* was 87.5%, but the sun- and oven-dried sea cucumbers had moisture content below 10%. According to Ozer *et al.* (2004) and Aydin *et al.* (2011), the high-water content in fresh products is due to the water bound to the cell wall and protoplasm.

In the present work, we found relatively no difference in the HPLC and FTIR profiles of sea

1 cucumber extracts dried by different techniques. FTIR analysis indicated that during drying, the bioactive compounds contained in sea cucumber extract were stable. There was no change in the active functional group. This showed that there were no damages on the cytotoxic compounds in sea cucumber extract during boiling and drying. In other words, the bioactive compounds were stable to heat and photo-oxidation. Therefore, saponins, one of the bioactive compound groups responsible for their bioactivity, also did not change. This was confirmed by bioactivity tests which showed that the extracts dried under the sun and oven had the same cytotoxicity against T47D cells as compared to fresh sea cucumber extracts. This is in accordance with Barakat *et al.* (2015) who found that the aglycone group in saponins was stable to heat, and did not completely degrade during heating.

Many saponins do not have UV absorption; so, there is a possibility of bioactive compounds not being detected with an HPLC detector. Therefore, in the future, the detection of saponin variations found in *H. atra* during drying should be carried out using more sensitive instruments, such as LC-MS/MS or NMR. In addition, saponins in *H. atra* also have complexity and diversity, some of which may have not been fully elucidated. Saponins in *H. atra* that have been identified include holothurin, echinoside, and calcigeroside (Grauso *et al.*, 2019).

In a study by Caulier *et al.* (2013), the saponin compound in *H. scabra* was stable even though it had passed postharvest processing. It was further said that saponins were more stable if the compounds were still in sea cucumber tissue. Sea cucumber processing is generally carried out by washing, boiling, salting, and drying. In the present work, the stability of saponin compounds in the sea cucumbers will benefit the process of developing bioactive saponins from sea cucumbers as a source of medicinal compounds. As is known, many bioactive compounds from marine environments are unstable and easily damaged during the postharvest handling process.

Conclusion

The drying of sea cucumber (sun- and oven-drying) did not affect the cytotoxicity of its ethanolic extracts against the human breast ductal carcinoma (T47D) cells. The HPLC and FTIR data showed that the bioactive compounds contained in the ethanolic extract remained stable during drying.

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Their bioactivities include as an immunostimulant,antiangiogenic, anticancer, anti...

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induction of apoptosis and caspase-3 activation inT47D breast cancer cells. The bi...

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Active fraction analysisThe analysis of active fractions used as reference was perf...

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100 × 2.0 mm columns, a flow rate of 0.2 mL/min, and an elution system starting w...

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different drying techniques is shown in Figure 1. Foruntreated cells, they maintaine...

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TSATSBPMPOAPOB

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FTIR profilesMetabolite profile analysis of sea cucumber extracts obtained from dif...

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4540POA35%T30252015TS1050.04000.036003200280024002000180016001400...

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2.8 µg/mL), the HPLC of this fraction was then used as a reference to detect the pr...

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minFigure 4. HPLC chromatograms of sea cucumber ethanolic extracts dried thro...

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