RESEARCH ARTICLE

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The Ethyl Acetate Fraction of Marine Sponge *Stylissa carteri* Induces Breast Cancer Cell Death via Upregulation of Mcl-1S: an *In vitro* Study

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Abstract

Objective: To evaluate the potency of the fraction of marine sponge *Stylissa carteri* in inducing cell death, inhibiting spheroid growth, and its impact on pro-apoptotic protein Mcl-1S in breast cancer cells. **Methods:** *Stylissa carteri* were collected from Pramuka Island followed by ethanol extraction and ethyl acetate fractionation. To evaluate the cytotoxic effect of fraction, the HCC-1954, MDA MB 231, and MCF-7 cells were treated with the fraction of *Stylissa carteri* and MTT assay was then performed. The effect on spheroid growth was evaluated in HCC-1954 cells. The combined effect of the ethyl acetate fraction and paclitaxel were analyzed using combination index (CI) and immunoblotting on the pro-apoptotic protein Mcl-1S. Furthermore, compounds in this fraction were identified using GC-MS. **Results:** Data showed that both the MDA MB 231 and HCC-1954 cells were interestingly more sensitive to the fraction as compared with MCF-7 cells. The IC_{50} of the ethyl acetate fraction on HCC-1954, MDA MB 231 and MCF-7 were 4.1 µg/ml, 3.9 µg/ml, and 123.8 µg/ml, respectively. In addition, the fraction triggered spheroid destruction within 10 days. The CI of paclitaxel and ethyl acetate fraction of *Stylissa carteri* were less than 0.52. Moreover, this combination induced upregulation of the Mcl-1S protein. Furthermore, some fatty acid-based structures were predicted as the major compounds in this fraction. **Conclusion:** The ethyl acetate fraction of *Stylissa carteri* induces cell death and spheroid destruction in aggressive breast cancer cells. It has a synergistic cytotoxic effect with paclitaxel on MDA MB 231 cell death and upregulates Mcl-1S protein.

Keywords: Cytotoxicity- marine product- pro-apoptosis- spheroid- triple negative breast cancer

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Introduction

Breast cancer (BC) is the most common cancer in women worldwide. More than 2.2 million women each year suffer from BC annually, with an estimated death rate of 684,996 in 2020 in the world (Sung, et al., 2021). Among BC subtypes, triple negative breast cancer (TNBC) and HER2-positive BC are the most aggressive types of BC and is associated with a higher mortality rate than other BC subtypes (Brewster et al., 2014; Tian et al., 2017; Johansson et al., 2019).

TNBC refers to BC type that does not overexpress the genes for the estrogen receptor (ER), progesterone receptor (PR) and receptor tyrosine-protein kinase erbB-2 (HER2) implicating that this type has no well-defined molecular targets. The cytotoxic chemotherapy is currently the only pharmacological treatment option for TNBC (Palma et al., 2015; Tian et al., 2017). One of the major cytotoxic chemotherapy in TNBC is a taxol based regimen including paclitaxel (Tian et al., 2017). Paclitaxel works by binding to the n-terminal of the tubulin subunit region, thereby increasing the stability of microtubules that are resistant to depolymerization. Paclitaxel inhibits its breakdown into tubulin, so that the cell cycle will stop at the G2/M phase and inhibit cell proliferation (Weaver, 2014; Kampan et al., 2015). Nevertheless, paclitaxel is less effective in the advance stage of TNBC.

Furthermore, HER2 is member of epidermal growth

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factor (EGFR) family whom its amplification results in activation of tyrosine kinase that initiate MAPK and PI3k/Akt signaling cascade. The signaling cascades amplified by HER2 are responsible for cell differentiation, migration, cell cycle control, angiogenesis, and apoptosis avoidance (Lee-Hoeflich et al., 2008; Loibl and Gianni, 2017; Hsu and Hung, 2018; Patel et al., 2020). Therefore, HER2 BC patients are treated with HER2 inhibitors (e.g trastuzumab, lapatinib, pertuzumab). Unfortunately, about two third of HER2-positive BC patients are not well response to trastuzumab. Different mechanism of resistance have been identified (Patel et al., 2020).

A novel drug discovery for TNBC as well as HER2-positive BC patients is necessary to achieve better BC patient survival. One of the promising sources is from marine sponges one of which is *Stylissa carteri*. It is predominantly found along the Red Sea to Australia (Boohaker et al., 2012). Recent studies indicated that there are at least 15 alkaloids in *Stylissa carteri* (Afifi, 2017; Hamed et al., 2018). *Stylissa carteri* that was obtained from the island of Bangka North Sulawesi showed strong activity against HeLa cell lines and HCT-116 colon cell lines. The ethyl acetate fraction from ethanol extract *Stylissa carteri* was able to inhibit the activity of proteasome E2 (Ubc13) and the interactions between p53-MDM2 (E3) (Afifi, 2017). Our previous study showed the ethanol extract of *Stylissa carteri* has a cytotoxic effect in different BC cell lines including luminal A (MCF-7), HER2+ (SKBR3, HCC-1954) and TNBC (MDA MB 231), and also having an effect in cervical cancer cells (Hardani et al., 2018; Bashari et al., 2019). Moreover, it inhibits MDA MB 231 cell proliferation and migration (Bashari et al., 2019).

Resistance to apoptosis is one of the characteristics of TNBC as well as HER2-positive BC cells (Karakas et al., 2018; Kamalabadi-Farahani, et al., 2019). Interfering anti-apoptotic protein is one of the promising and challenging mechanisms for cancer therapy (Hassan et al., 2014). One of the proteins that play a role in the process of apoptosis is Mcl-1 (Seo et al., 2011; Wong, 2011; Elumalai et al., 2012). Myeloid cell leukemia 1 (Mcl-1) is a protein from the Bcl-2 family which plays a pivotal role as apoptotic regulator. The Mcl-1 protein has two variants namely the Mcl-1L (long) anti-apoptotic protein and the Mcl-1S (short) pro-apoptotic protein. Mcl-1S has a pro-apoptotic effect in the same way as other BH3 proteins, by binding to the anti-apoptotic Bcl-2 protein (Bae et al., 2000).

Further research on the potential of *Stylissa carteri* as an anti-cancer has become an opportunity to explore the combination of BC therapy. This study was aimed to determine the cytotoxic effect of a single or combination administration of ethyl acetate fraction of *Stylissa carteri* with paclitaxel and to determine its effect on the Mcl-1S protein expression in breast cancer cells.

Materials and Methods

Chemicals and reagents

Fetal bovine serum (FBS) (cat No. 10270106), RPMI 1640 medium (cat No. 11875093), and penicillin streptomycin (cat No. 15140122) were obtained from Gibco, USA. The primary antibodies of Mcl-1S (cat No. PA5-27597), α-tubulin primary antibody (cat No. 62204), and HRP secondary antibody (cat No. M32207) were bought from Invitrogen. Dimethyl sulfoxide (DMSO) (cat No. D8418), and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (cat No. M2128) were purchased from Sigma-Aldrich, USA. Paclitaxel was from Pharmacy of Hasan Sadikin Hospital, Bandung, Indonesia. The other chemicals were bought from Merck, USA.

Fractionation of Stylissa carteri

Marine sponge *Stylissa carteri* was taken from Pramuka Island, Kepulauan Seribu National Park, Jakarta at a depth of 10 meters in 2017. The sponge was collected and identified by Mr. Beginer Subhan from Bogor Agricultural Institute. An 800 g fresh sponge was obtained and then chopped and macerated directly with ethanol before going through the extraction process. Through the extraction process 10.2 g of powder was obtained from ethanol extract. Fractionation was carried out on 5 g of extract powder using n-hexane followed by ethyl acetate as the solvent. The ethyl acetate fraction was then evaporated to obtain dry powder. They were diluted with DMSO to get a concentration of 40,000 µg/ml as the stock solution. Next, the solution was diluted with RPMI for the indicated concentrations.

Cell culture and conditions

The MCF-7, HCC-1954 and MDA MB 231 cells were used to evaluate the activity of the fraction of Stylissa carteri. MCF-7 cell are the Luminal breast cancer subtype. HCC-1954 cells are HER2 breast cancer subtype while MDA MB 231 cells are the TNBC subtype. The MDA MB 231 cells were obtained from Dr. Thordur Oskarsson (DKFZ, Germany), HCC-1954 cells were from Prof. Stefan Wiemann (DKFZ, Germany), MCF-7 cells were from Prof. Ahmad Faried and Prof. Hiroyuki Kuwano. These cell lines were cultured in RPMI 1640 medium enhanced with 10% FBS, 1% penicillin/streptomycin at 37^{o}C with 5% CO_2.

Cytotoxicity assay

To evaluate cytotoxic activity in BC cells we used MTT assay. Cells were seeded in 96 well plate, incubated for 24 hours, treated with serial concentrations of tested materials for 72 hours. A culture medium containing 1% DMSO was used as control. Next, the cells were then given MTT solution for 4 hours. DMSO solution was added to stop the reactions and to dissolve the formazan crystal. Finally, the absorbance was recorded at 550 nm wavelength using plate reader. The assay was carried out with 3 replicates for each culture.

Spheroid growth assay

HCC-1954 spheroids were generated according to previous study (Bashari, et al., 2021). In brief, the HCC-1954 cells were seeded on agarose-coated (Sigma Aldrich, Steinheim, Germany) 96-well plates. For spheroid formation, the plates were centrifuge and incubate for 4 days. Next, spheroids were exposed or not exposed to

ethyl acetate fraction of Stylissa carteri. Spheroids were captured at day 0, 4th, 9th and 10th of intervention using camera on inverted microscope. Images were analyzed using ImageJ software to have ferret diameter.

Combination index

The combination index (CI) was obtained by analyzing the cytotoxic effect of different concentrations of the ethyl acetate fraction of *Stylissa carteri* alone or in combination with paclitaxel in MDA MB 231 cells. Combination index (CI) was generated with Compusyn software based on Chou Talalay method (Chou, 2010).

Western Blot

All sample cells were washed three times with phosphate-buffered saline (PBS) and lysed. The whole cell lysates were separated by 10% SDS-PAGE followed by transferred onto super nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBS at room temperature for 1 hour followed by incubation with the primary antibodies (Mcl-1S) (1:300) and then with horseradish peroxidase-conjugated secondary antibodies with dilution of 1: 1,000. The α-tubulin (1: 300) was used as protein loading control. The antibody-antigen binding was detected using ECL chemiluminescence and observation using film.

GC-MS analysis

The sample was analyzed using Shimadzu QP2010 Ultra Gas Chromatography with Mass Spectrometer as a detector. The type of MS detector was a single quadrupole (Vetvicka, and Vetvickova, 2016). A Shimadzu Reverse phase column (RP-5 non-polar column 30mm x 0.25mm x 0.25µm film thickness) was used for separation in the GC system. The MS mode was set at 70 eV ionizing energy, scanning from m/z 1 to 2,000 at 0.3 scan/sec. The temperature of the ion source and the quadrupole were set at 300°C and 280°C, while the electron multiplier voltage was set at 0.8kV. GC-grade helium (purity 99.999%) was used as carrier gas at a flow rate of 1 mL/min and separation ratio was set to 400:1. The chromatographic conditions were set as commonly used for gas chromatographic analysis. The column temperature was set at 40°C and maintained for 10 min. Then after 56 min, it was increased to 180°C (rate 2,5°C/min), and finally maintained at a rate of 20 minutes. 0.2µL was taken from the sample for Injection volume. The identification of compounds, retention time and retention index were checked with a database from NIST11 Mass Spectral Library software.

Statistical analysis

The five-parametric-logistic regression model by GraphPad Prism ver. 9 (GraphPad Software, LCC) was used to generate the drug curves and to determine the IC_{50} values. The spheroid growth assay is for assess the growth inhibition effect. The minimal level of significance was $p<0.05$.

Results

The Ethyl Acetate Fraction of Stylissa carteri showed potent cytotoxic activity in HCC-1954 and MDA MB 231 cells, but not in MCF-7 cells

MTT assay was performed to evaluate the cytotoxic effect of the ethyl acetate fraction of *Stylissa carteri* in MCF-7, HCC-1954, and MDA MB 231 cells. Data revealed that the fraction of *Stylissa carteri* stimulated cell death in all cell lines in a dose-dependent manner (Figure 1). Interestingly, the IC_{50} of the ethyl acetate fraction of *Stylissa carteri* showed high efficacy in HCC-1954 and MDA MB 231 cells and but not in MCF-7 cells, with an IC_{50} value of 4.1 µg/ml, 3.9 µg/ml and 123.8 µg/ml, respectively. Therefore, for further study, we focused on the ethyl acetate fraction of *Stylissa carteri* on HCC-1954 cells and MDA MB 231 cells.

The ethyl acetate fraction of Stylissa carteri induces destruction of spheroids of HCC-1954 cells

The spheroid growth assay was conducted in HCC-1954 cells upon treatment of ethyl acetate fraction of Stylissa carteri. Interestingly, the treated spheroids

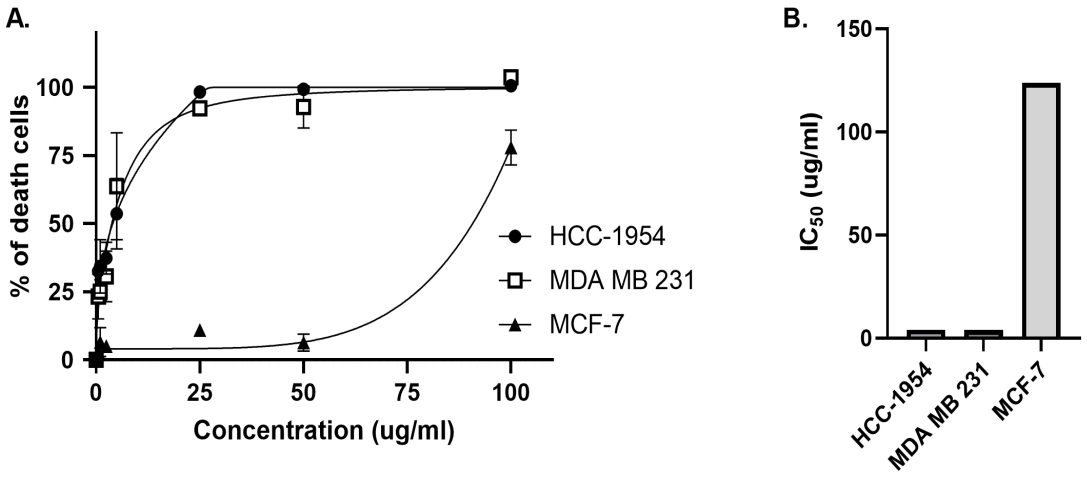

Figure 1. Cell Death Curve Showing the Percentage of MCF-7, HCC-1954 and MDA MB 231 Cells Death upon a Serial Dilution of Fractions of *Stylissa Carteri*. The IC_{50} as well as the cell death curve were calculated and analyzed using five-parametric logistic regression.

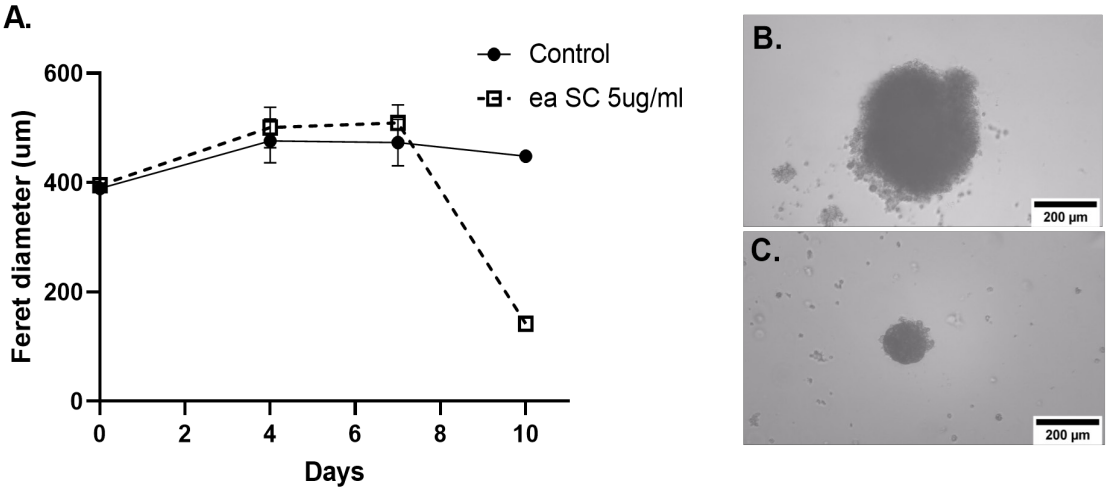

Figure 2. The Ethyl Acetate Fraction of *Stylissa carteri* Damaged Spheroid of HCC-1954 Cells in 10 Days. Spheroids were treated with 5µg/ml of ethyl acetate fraction of *Stylissa carteri* or untreated. Spheroid ferret diameter were declined upon treatment. Represented spheroids on day 10th were control (B) and treated group (C). Data was shown as mean and SD of 7 replicate each group.

were destroyed within 10 days of treatments (Figure 2).

The ethyl acetate fraction of Stylissa carteri induces synergistic cell death with paclitaxel in MDA MB 231 cells

The combination test was carried out in the same manner as the cytotoxic test using the MTT assay method, MDA MB 231 cells were exposed to a combination sample of the ethyl acetate fraction *Stylissa carteri* and paclitaxel with different concentrations. Based on the compusyn analysis, all combinations between ethyl acetate fraction *Stylissa carteri* and paclitaxel showed synergistic activity on inducing MDA MB 231 cell death (CI <1) (Figure 3).

The Mcl-1S Protein expression in MDA MB 231 cells upon treatment of the Ethyl Acetate Fraction of Stylissa carteri (SC) and paclitaxel (PTX)

Mcl-1S protein expression was detected using the Western blot analysis. Data showed that the ethyl acetate fraction of *Stylissa carteri* induced upregulation of Mcl-1S. Interestingly, Mcl-1S was more prominent upon combination with paclitaxel (Figure 4).

GC-MS Data of the Ethyl Acetate Fraction of Stylissa carteri

The results of GC-MS testing of the ethyl acetate fraction of *Stylissa carteri*, showed several peaks on the chromatogram but there were three peaks of the dominant compound (Figure 5). The three peaks were identified as compounds 2-Ethyl Hexanol, 1-Dodecanol, 9,12-Octadecadienoic acid/linoleic acid. Based on chromatogram, the ethyl acetate fraction of *Stylissa carteri* was dominated by fatty acid compounds. The other predicted compounds are shown in Table 1.

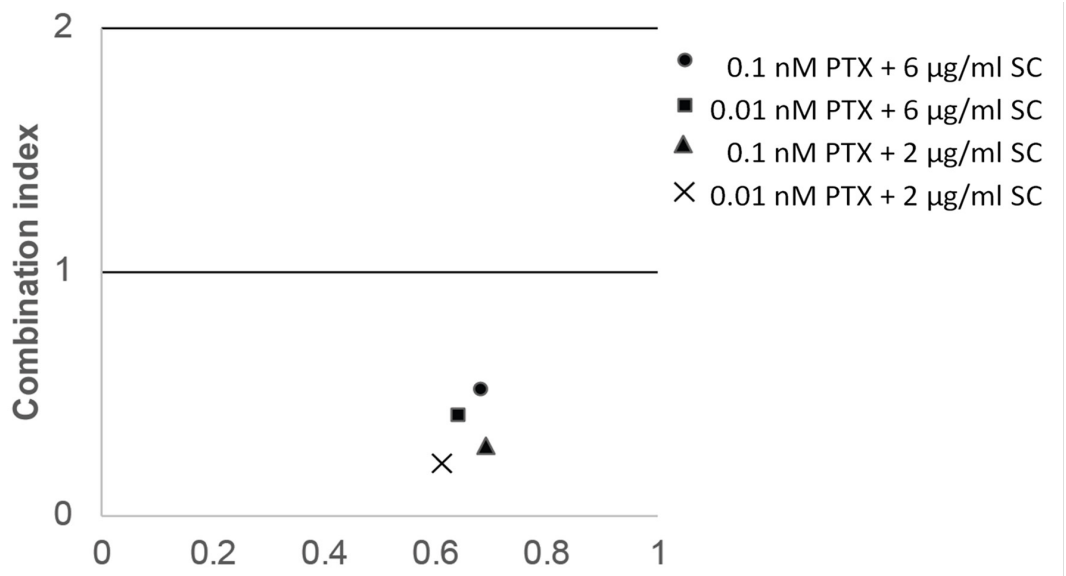

Figure 3. Combination Index (CI) of the Cytotoxic Effect of the ethyl acetate Fraction of *Stylissa carteri* (SC) and Paclitaxel (PTX)

Table 1. GC-MS Profile of the ethyl Acetate Fraction of *Stylissa carteri*

Compound name	Peak#	R.Time	Area%	Height%	A/H
Ethylbenzene	1	3.001	0.29	0.75	7.72
1-Hexanol, 2-ethyl-	2	5.248	1.26	6.43	3.95
Benzene, 1,2,3,4-tetramethyl-	3	6.641	0.07	0.24	6.23
1,2-Benzenediol	4	7.862	1.5	2.5	12.08
1-Undecene, 4-methyl-	5	9.225	0.04	0.14	5.7
Benzofurane-7-carboxylic acid, 2,3-dihydro-5-b	6	9.395	0.16	0.43	7.37
Hexanoic acid, 2-ethyl-, anhydride	7	10.445	0.02	0.07	5.21
1H-Cycloprop[e]azulene, decahydro-1,1,7-trime	8	10.513	0.18	0.26	13.99
Hexadecanal	9	10.745	0.02	0.13	3.1
Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethy	10	11.147	0.2	1.04	3.8
1-Dodecanol	11	11.594	0.84	6.43	2.62
Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimeth	12	12.02	0.43	1.96	4.44
Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dime	13	12.195	0.06	0.25	4.42
Diethyl Phthalate	14	12.62	0.87	2.62	6.68
Diethyl Phthalate	15	12.72	1.02	2.08	9.85
Diethyl Phthalate	16	13.002	1.46	2.93	10.07
Cyclooctasiloxane, hexadecamethyl-	17	13.37	0.01	0.07	2.09
1-Oxaspiro[4.5]decan-2-one, 6-isopropenyl-9-m	18	13.57	0.01	0.03	4.51
Octacosane	19	13.867	0.14	0.24	11.89
1-Tetradecanol	20	14.043	0.99	1.86	10.71
Naphthalene, 1,6-diacetyl-	21	14.595	0.04	0.16	4.97
Hexadecane	22	14.767	0.08	0.22	7.27
Hexadecane, 1-iodo-	23	14.981	0.07	0.13	11.59
Hexadecane	24	15.389	0.2	0.72	5.48
Isopropyl Myristate	25	15.62	0.03	0.13	4.16
Hexadecane	26	15.824	0.03	0.12	4.75
Phthalic acid, bis(7-methyloctyl) ester	27	16.043	0.07	0.22	6.11
Hexadecane, 1,1-bis(dodecyloxy)-	28	16.253	0.2	0.52	7.59
Cyclohexanone, 3-(3,3-dimethylbutyl)-	29	16.397	0.18	1.03	3.57
Hexadecanoic acid, methyl ester	30	16.695	0.31	0.87	7.26
Hexadecane, 7-methyl-	31	16.845	0.06	0.15	8.46
l-(+)-Ascorbic acid 2,6-dihexadecanoate	32	17.073	0.61	1.67	7.37
l-(+)-Ascorbic acid 2,6-dihexadecanoate	33	17.496	0.66	1.19	11.22
D-Gulonic acid, .gamma.-lactone, cyclic 2,3:5,6	34	18.07	0.06	0.1	11.91
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	35	18.324	0.57	2.93	3.91
9,12-Octadecadienoic acid (Z,Z)-	36	18.746	10.96	29.2	7.56
9,12-Octadecadien-1-ol, (Z,Z)-	37	19.798	0.13	0.47	5.61
Hexadecanoic acid, 1-(hydroxymethyl)-1,2-etha	38	20.145	0.05	0.37	2.54
Cyclononasiloxane, octadecamethyl-	39	20.42	0.06	0.44	2.99
9,12-Octadecadienoyl chloride, (Z,Z)-	40	21.601	0.26	1.4	3.71
Oleic acid, (2,2-dimethyl-1,3-dioxolan-4-yl)me	41	21.796	0.09	0.2	9.04
Ethanol, 2-(9,12-octadecadienyloxy)-, (Z,Z)-	42	22.303	0.17	0.21	16.6
E,Z-1,3,12-Nonadecatriene	43	23.366	0.28	0.86	6.65
Cyclononasiloxane, octadecamethyl-	44	23.52	0.13	0.63	4.28
Tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16	45	25.87	15.12	4.67	65.19
Trilinolein	46	26.328	9.25	5.4	34.53
9-Octadecenoic acid (Z)-, 2-hydroxy-3-[(1-oxo	47	29.964	38.19	5.44	141.48
Oxirane, hexadecyl-	48	30.738	1.53	2.9	10.63
	49	31.97	7.66	1.95	79.09
	50	32.247	1.07	1.66	13.01
	51	32.52	1.31	1.01	26.1
Propyleneglycol monoleate	52	33.916	0.27	0.28	19.56
Tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16	53	34.296	0.72	2.27	6.4

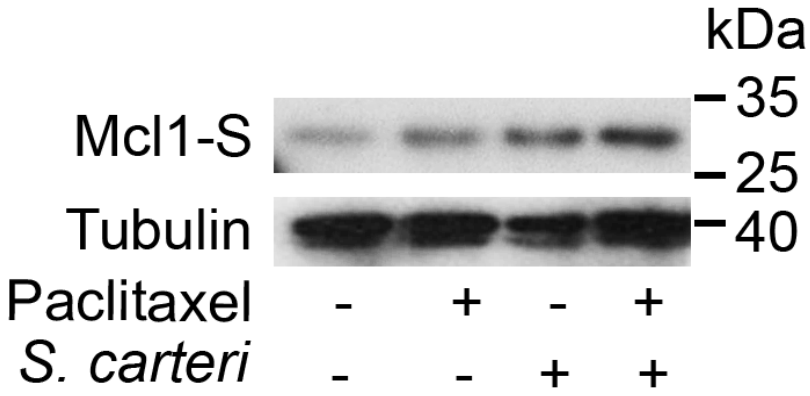

Figure 4. Upregulation of Mcl-1S Protein Upon Treatment with Combination of the ethyl Acetate Fraction of *Stylissa carteri* and Paclitaxel. Tubulin was used as loading control.

Discussion

Stylissa carteri is a sea sponge located spread across the Red Sea to Australia including Indonesia (Boohaker et al., 2012). There have been several studies regarding *Stylissa carteri* as an anti-cancer. Our previous study showed the ethanol extract of *Stylissa carteri* has a cytotoxic effect in different BC cell lines including luminal A (MCF-7), HER2+ (SKBR3, HCC-1954) and TNBC (MDA MB 231), and also having an effect in cervical cancer cells (Hardani, et al., 2018; Bashari, et al., 2019). Moreover, it inhibits MDA MB 231 cell proliferation and migration (Bashari, et al., 2019).

Studies showed that *Stylissa carteri* contains several compounds including alkaloids, flavonoids, triterpenoids, steroids and peptides (Afifi, 2017; Dewi, 2017). Our GC-MS chromatogram result showed ethyl acetate fraction of *Stylissa carteri* is dominated by fatty acid compounds. The three peaks were identified as compounds 2-Ethyl Hexanol, 1-Dodecanol, 9,12-Octadecadienoic acid/linoleic acid (Figure 5).

This is supported by other study that successfully isolated several compounds from *Stylissa carteri*, including Stylissamide A, Stylissoside A, and 9,10,11-Trihydroxy-(12Z) -12-Octadecenoic acid. The three compounds were reported to have a fatty acid structure in their structural framework. Especially in compounds 9,10,11-Trihydroxy-(12Z) -12-Octadecenoic acid, which is a fatty acid compound (linoleic acid) substituted with three OH groups (hydroxy) on carbon atoms number 9, 10 and 1. Stylissamide A and Stylissoside A compounds were reported having fatty acid side chains with the length of the carbon chain in the fatty acid component of the Stylissamide A compound (15 carbon atoms) is shorter than that of the Stylissoside A compound (21 carbon atoms). Based on the results of studies in silico (modeling and docking simulations) the longer carbon chains in fatty acid components will cause the bonding instability of complex compounds by sliding the ligand from the main binding site (Abdelhameed, et al., 2020).

Stylissamide A and Stylissoside A compounds have molecular structures like ceramide compounds, which contain long sphingoid chains (Sph) in the main structural framework and bind to fatty acid molecules via amide bonds as side chains. Ceramide compounds are also reported as potential anticancer agents. In preclinical studies, in addition to increasing water solubility, long chain fatty acids from the original ceramide structure are often replaced with short chain fatty acids (acetic acid, hexanoic acid and octanoic) to form compounds C2-, C6-, and C8-Ceramide, thus causing cell exposure to ceramide compounds with short chain fatty acids producing anticancer activity. The double bond on carbon number 4 and 5 of the sphingoid long chain component in the ceramide compound can inhibit PP2Ac/PP2A so that it inhibits apoptotic activity in cancer cells. In the long chain sphingoid component of the compounds Stylissamide A and Stylissoside A there are no double bonds in carbon numbers 4 and 5 and this affects both anticancer activities. PP2Ac / PP2A has tumor suppressor function by triggering apoptosis in different cancer cells (Liu, et al., 2013).

This study was conducted to explore the effect of the

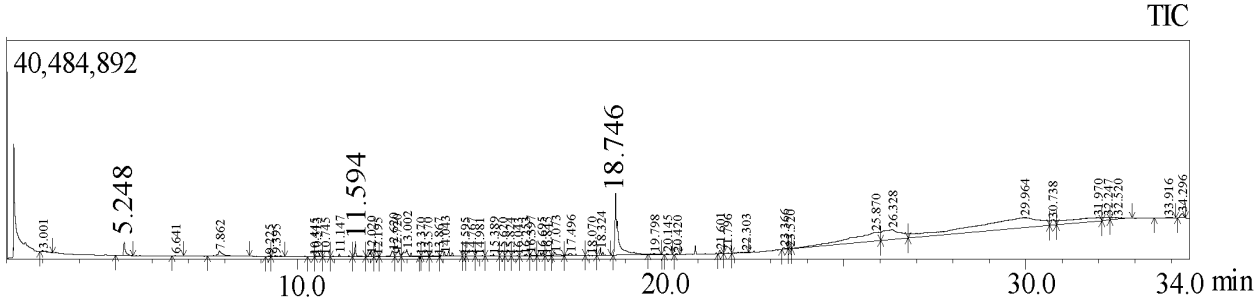

Figure 5. GC-MS Chromatogram of the ethyl Acetate Fraction of *Stylissa carteri*

Stylissa carteri sponge in breast cancer cells. In this study, a small IC_{50} values obtained from the ethyl acetate fraction of *Stylissa carteri* exposed to HCC-1954 and MDA MB 231 were 4.1 µg/ml and 3.9 µg/ml, respectively. Moreover, a big IC_{50} values obtained from the ethyl acetate fraction of *Stylissa carteri* exposed to MCF-7 were 123.8 µg/ml (Figure 1). It is indicating that the ethyl acetate fraction of *Stylissa carteri* has potential activity on the aggressive cells HER2 and TNBC subtype. A compound can be categorized as a potential cytotoxic compound if the IC50 value is smaller than 20 µg/ml (Sajjadi, et al., 2015). Nevertheless, further study needs to confirm the specific effect using other cell line including the normal cell line.

Other studies showed that the alkaloid compounds contained in *Stylissa carteri* become protein kinase inhibitors that play a role in cell proliferation (Patel, et al., 2010). The ethanol extract of *Stylissa carteri* inhibits cell proliferation and induces apoptosis in TNBC cells cultured in three-dimensional cells (Bashari, et al., 2019). Similarly, the ethyl acetate fraction of *Stylissa carteri* triggered destruction of spheroid of HCC-1954 cells within 10 days (Figure 2).

It has been known that cancer cells activate several pathways to avoid cell death. Therefore, it is rational to target cancer cells by combining different agents which have different mechanisms. Previous studies had shown the synergistic effect of combination of ethanol extract of *Stylissa carteri* and paclitaxel in triggering MDA MB 231 cell death (Bashari, et al., 2019). Similarly, in this study, the ethyl acetate fraction of *Stylissa carteri* was combined with paclitaxel. Paclitaxel is one of the main regimens in the treatment of breast cancer. Paclitaxel works in the G2-M phase, causing cells to stop at that phase and inhibit cancer cell proliferation (Weaver, 2014; Liu, et al., 2015). Our study revealed that the combination between the ethyl acetate fraction of *Stylissa carteri* and paclitaxel in MDA MB 231 cells has synergistic effect on cytotoxicity of MDA MB 231 cells (Figure 3).

Furthermore, previous study showed that the isolated compound from *Stylissa carteri* inhibit the activity of ubiquitin-proteasomes (Ubc13) in cancer cells (Afifi, 2017). Proteasome is a protein complex that is responsible for intracellular protein degradation. The ubiquitin-proteasome (UPS) system controls almost all basic cellular processes such as cell cycle development, signal transduction and cell death (Tanaka, 2009; Afifi, 2017). One of the important substances of ubiquitin-proteasomes is p53. If p53 were accumulated under inhibition of ubiquitin-proteasomes, the activity of p53 will be increased, thus anti-cancer effects are provided. If p53 is active, its role as a tumor suppressor gene can read the damage signal from within the cell so that it will activate the apoptotic pathway intrinsically (Orlowski, 1999; Thomas et al., 2010).

Peptides contained in *Stylissa carteri* play a role in the regulation of pro-apoptotic proteins such as BAX and BAK. Increased expression of pro-apoptotic proteins induces apoptosis or cell death (Patel et al., 2010). Interestingly, in this study it showed the ethyl acetate fraction of *Stylissa carteri* induced upregulation of Mcl-1S. Moreover, Mcl-1S was more prominent upon combination with paclitaxel (Figure 4). Mcl-1S is a pro-apoptotic protein (Bae et al., 2000; Kim and Bae, 2013). Other study showed that Mcl-1S inhibit mitotic phase and cause DNA damage accumulation (Streletskaia et al., 2020).

In conclusion, the ethyl acetate fraction of *Stylissa carteri* has a cytotoxic effect on the aggressive BC cells, MDA MB 231 and HCC-1954 cell death. The combination of paclitaxel and ethyl acetate fraction of *Stylissa carteri* has a synergistic effect in inducing death of MDA MB 231 cells. The ethyl acetate fraction of *Stylissa carteri* and paclitaxel cause an increase in the Mcl-1S protein in MDA MB 231 cells, suggesting activation of apoptosis as the underlying mechanism. Further studies need to be conducted to identify which of the compound play important role for the finding. Knowing the ethyl acetate fraction of *Stylissa carteri* has a potent cytotoxic effect on the aggressive BC cells, indicating that the active compound interfere similar target in both cell types.

Author Contribution Statement

The authors confirm contribution to the paper as follows: study conception and design: Muhammad Hasan Bashari, Eko Fuji Ariyanto; data collection: Yudhi Aulia, Ajeng Kartika Sari, Tenny Putri, Nurul Qomarilla, Harold Atmaja; analysis and interpretation of results: Muhammad Hasan Bashari, Ikhwan Resmala Sudji; draft manuscript preparation: Muhammad Fadhil, Agnes Rengga Indrati, Enny Rohmawaty, Eko Fuji Ariyanto. All authors reviewed the results and approved the final version of the manuscript.

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Statement conflict of Interest

No potential conflict of interest was reported by the authors.

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