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Assessments of anti-breast cancer activity and profiling of active compounds using LC-MS/MS from the Indonesian *Agelas nakamurai*

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ABSTRACT

Breast cancer is now one of the leading causes of death in women worldwide. Finding a cure is, therefore, critical for women's health. Marine sponge-derived drugs have gained interest in breast cancer treatment. The development of marine natural products into cancer therapy includes several stages. It starts with the screening using the cytotoxic test method, a preclinical trial using the animal mode, clinical trial phases I and II. In this study, we focus on the basic cytotoxic test to find the new source of anti-breast cancer compounds derived from *Agelas nakamurai* to know the potential for further investigation to become lead compounds. However, the anti-breast cancer potential of the Indonesian marine sponge *A. nakamurai* has never been studied. This study aimed to evaluate the anti-breast cancer potential of extract and fraction of *A. nakamurai* against epithelial human breast cancer cells (MDA-MB-231 and MCF-7 cells line) and to preliminary profile the known active compounds. The maceration using methanol was used for extraction and followed by partitioning with varied solvents [ethyl acetate (EtOAc), butanol, and water]. Fractionation was done using normal phase open column chromatography, and the liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) was used for analyzing compounds. MDA-MB-231, MCF-7, and HEK-293 cell lines were used for biological activities. The chromatographic separation of the EtOAc fraction led to the F7 subfraction which strongly inhibits proliferation against the MDA-MD-231 (IC_{50}: 10.677 µg/mL), MCF-7 (IC_{50}: 15.154 µg/mL), and HEK-293 cells line. The LC-MS/MS data of active fraction F7 contained agelasin-D and ageloxime-D. This study reported that fraction 7 of *A. nakamurai* showed strong activity against MDA-MB-231, MCF-7, and HEK-293 cell lines. Further analysis will be carried out to know the mechanisms of action of active compounds.

INTRODUCTION

Breast cancer has the highest prevalence in the world for women. Mostly for East Asia and African countries, there were 27/100,000 incidents [1]. Several government policies related to the handling of this cancer include the socialization of healthy living, early detection, improvement of health facilities, and the availability of drugs and vaccines. Currently, the availability of imported cancer drugs is very limited at prices that are not affordable for the lower middle class. This is a very crucial issue. The development of cancer drugs, both herbal and modern drugs for chemotherapy, is a priority. Developing cancer therapy from natural resources needed several steps as follows: basic screening to evaluate the cytotoxic effect on the targeted cancer cells, then a preclinical trial using an animal model, and finally, the full clinical test [2]. Cancer drug development research focuses primarily on terrestrial bioresources such as plants and microorganisms, with little attention paid to marine resources. The study of anticancer drugs in marine

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invertebrates differs from that of plants in that some plants can be used as herbal medicine, whereas studies of marine invertebrates are primarily interested in finding active lead compounds. Investigating natural products for cancer therapy is very important since 60% of cancer drugs were first found in nature [2]. Researchers studying natural products have discovered that marine organisms can produce active compounds. Between 1985 and 2012, 16,617 new compounds were discovered in marine organisms. Around 4,196 or 25.25% were active compounds, with 56% being anti-cancer [3]. Until 2016, 1,277 new compounds were isolated from marine organisms, 430 of which were from Indonesian sponges and belonged to 40 different genera [4].

Agelas nakamurai is a powerful sponge that belongs to the Agelasidae family and the genus *Agelas*. Several species in this genus are reported to contain brominated 2-pyrrole carboxylic acid derivatives and unique terpenoid derivatives, such as the sesquiterpene derivatives hypotaurocyamines and adenine derivatives [5]. Several compounds have been identified in *A. nakamurai*, including 4-(4,5-dibromo-1-methyl-1H pyrrole-2-carboxamido) butanoic acid, agelasin A-D, midpacamide, methyl-4,5-dibromo1-methyl-1H-pyrrole2-carboxylate, dibromohydroxyphakellin HCl, dibromophakellin HCl, mucanadin C, agelasine D-oxime, hymenidine, adenosine, 9-methyladenin, ageloxime D, longamide C [6], 2-guanidinoethanesulfonyl sesquiterpene, cyclohexylagelasidine A [7], elasidines B and C [8], nakamurols A–D [9], 2-oxoagelasiines A and F, 10-hydro-9-hydroxyagelasine F [10], and agelasines E and F [11]. Some of these compounds showed anti-cancer activity, such as agelasin-C, which demonstrated moderate activity against rat lymphoma cells (L5178Y), and cytotoxic activity against lung adenocarcinoma cells A549 with an IC_{50} of 16.7 µg/mL [12]. Interestingly the semisynthetic of ageladine A derivative by replacing the pyridine with azepine ring showed activity against DU145 prostate, A2058 melanoma, and MDA-MB-435 breast cancer cell lines. [13]. Slagenin B and C were bromopyrrole alkaloids isolated from the Okinawan *A. nakamurai* that showed cytotoxic activity against L1210 cells lines with IC_{50} 20.94 and 19.55 µM, respectively [14].

So far, there is no information on the activity of the substance isolated from the sponge *A. nakamurai* against MDA-MB-231 and MCF-7 breast cancer cells. This study will investigate potential anti-cancer substances derived from *A. nakamurai* against MDA-MB-231 and MCF-7. The methods used in this study were chromatographic separation, *in vitro* bioassay of the cytotoxic activity of *A. nakamurai,* and preliminary metabolic profiling using the liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS). In this study, the preliminary assessment of anti-cancers using cytotoxic tests was applied as the basic screening evaluation to decide further investigation, such as detailed structural determination of lead compounds, mechanism of action, optimizing compounds, and several preclinical and clinical tests.

MATERIALS AND METHODS

Sample characterization

The sponge samples used in this study were collected from the Biak area in 2020. The samples were collected by scuba diving from 5 to 10 m in depth. The fresh samples were immediately sent to the laboratory in cool boxes, where they were stored in Genomic Laboratory BRIN, Bogor, Indonesia, at freezer −20°C until they could be characterized and extracted. The sample with the code Spg-An-2020-Biak was prepared for spicule profiling by macerating the sponge tissue using sodium hypochlorite. After 15 minutes, the tissue was inverted in the Euromax microscope with 10× magnification. The characterization of the sample was done using the taxonomic method by referring to [15] and [16].

Extraction

The sponge samples were cut into cubes and freeze-dried for 24 hours. Extraction was done by maceration using methanol, followed by partitioning using ethyl acetate (EtOAc), butanol, and water as solvents. Following rotary evaporation, an anti-cancer screening was performed.

Separation and fractionation of anti-cancer active compounds

The following steps are taken to separate potential fractions: The fractions were separated using open column chromatography with a hexane-dichloromethane-ethyl acetate-methanol gradient system. The fractions were trapped in each band, removing the solvent and allowing the anti-cancer assay to proceed. In each fraction, the activity of MDA-MB-231 cells was evaluated.

Cytotoxic 3-45-dimethylthiazol-2-yl-25-diphenyltetrazolium (MTT) assay

Against HEK-293 cells line

The cytotoxic test of fractions and subfractions was carried out by the MTT method as proposed by Mosmann [17]. This test was based on the ability of the mitochondrial dehydrogenase enzyme in living cells to cleave the tetrazolium ring of the pale yellow MTT into purple formazan crystals.

The cells were counted and diluted as needed with culture media DMEM (Dulbecco's Modified Eagle Medium). Cells were then transferred into wells, 100 µL each, and incubated overnight. The media was discarded, the cells were washed with phosphate buffer saline (PBS), and then 100 µL of new media was added to the wells containing 25 µg/mL of *A. nakamurai* subfractions and incubated for 24 hours. After incubation, the media was removed, cells were washed with PBS, then MTT reagent was added in DMEM (5 µg/mL) at 100 µL/well and incubated for 4 hours. After that, a 10% sodium dodecyl sulfate stopper reagent was added in 0.01 N HCl, and then it was read with a microplate reader 595 nm and obtained an absorption indicating the absorbance of live 4T1 cells.

Against MDA-MB-231 and MCF-7 cell lines

The proliferation test of open column fractions was carried out with the following method.

To evaluate the antiproliferative effect of the fractions, the viability of the cells treated with *A. nakamurai* fractions was assessed for 24 hours using PrestoBlue™ cells viability reagent (Thermo Fischer Scientific, Waltham, MA). As much as 50 µL of cell suspension (about 1 × 10^4 cells) was seeded in 96 well plates (Thermo Fischer Scientific, Waltham, MA) and incubated overnight at 37°C. Fifty microliters of Dimethyl sulfoxide with various concentrations of *A. nakamurai* fractions were added to each well and incubated at 37°C for 24 hours. To measure the viability of the cells, 10 µL of the cells counting solution was added to each well and incubated at 37°C for 3 hours. Infinite M200 PRO microplate reader measured the absorbance at 535 nm with the reference wavelength at 560 nm (Tecan, Männedorf, Switzerland) 2 hours after the PrestoBlue™ cells viability reagent solution was given. The result was derived from triplicate experiments, and the relative cell proliferation inhibition (CPI) rate was calculated as a percentage by the following formula:

CPI rate (%) = (1–absorbance of the treated cells/ absorbance of the untreated cells) × 100.

LC-MS/MS analysis

LC-MS/MS Waters were used for analyzing active fractions. LC-MS/MS was carried out under the following conditions.

LC conditions: Solvent A: H_2O + 0.1% formic acid (FA), Solvent B: Acetonitrile + 0.1 FA, gradient system (95% A–100% B, flow rate 0.3 mL/minute, volume injection 1 µL, column: ACQUITY UPLC® BEH C8 1.7 µm 2.1 × 100 mm.

Mass conditions

Ionization type: ESI polarity: positive acquisition start time: 0.00 minute acquisition end time: 16.00 minute start mass: 50.00 *m/z* end mass: 1,200.00 *m/z* scan time: 0.100 seconds low CE: 6.00 eV high CE ramp start: 10.00 eV high CE ramp end: 40.00 eV cone mode: method settings cone voltage: 30 V collision mode: specific collision energy: 6.00 eV

RESULTS AND DISCUSSION

Characterization of sponge

The sponge (Spg-An-2020-Biak) is irregularly massive with a red color. The surface is smooth with uneven contour. The small osculum appears along the surface (Fig. 1). The texture is relatively compressible, especially when it is dry. The sponge consists of a network of fibers which are cored by spicules. The spicules are megascleres only, which are acanthostyle (around 200 × 10 µm) attached to the fibers. Based on the sponge-guided taxonomy handbook, the sponge is determined as *A. nakamurai*.

The evaluation of fractions against MDA-MB-231 cells line

Agelas nakamurai was macerated in MeOH to gain a wider compound group. Partition using semipolar solvent was applied, such as EtOAc, *n*-butanol, and water. This separation generated fractions that evaluated the activity against MDA-MB-231 (Table 1).

The result of evaluating the activity of fractions generated from the liquid–liquid partitions against the MDA-MB-231 cells line (Table 1) showed that semipolar solvent (EtOAc) fractions showed the strongest cytotoxicity. The results showed that the EtOAc fractions had IC_{50} values of 13.33 µg/mL. The previous study reported that the EtOAc fraction of *A. nakamurai* collected from Menjangan Island, Bali, Indonesia, did not show promising cytotoxic and protein kinase inhibitor activity. However, some of the isolated compounds agelasins A–D show active cytotoxicity against mouse lymphoma cells L5178Y with the IC_{50} less than 10.677 µg/mL [16]. Another screening test of *Agelas* extract from the Micronesian Ocean revealed mild activity against MDA-MB-231 breast cancer cells [18].

Separation of EtOAc fraction and its bioactivity profiling test

The EtOAc fraction was purified on a normal-phased open-column chromatography to give seven subfractions. Each subfraction was then tested in activity against MDA-MB-231 (Table 2).

The EtOAc fraction was then purified on normal-phased open-column chromatography to give seven subfractions. The extrapolation graphic data determined that IC_{50} of fraction 7 was 10.677 µg/mL (R^2: 0.988), and the EtOAc extract was 13.326 µg/mL (R^2: 0.977). When the cytotoxic activity of subfraction F7 against the MDA-MB-231 cells line was compared with EtOAc fractions, the open column subfractions F7 were stronger than the partition. This data demonstrated that the active ingredient's work was not synergistic. The single compound will contribute to the cytotoxicity.

In addition, the evaluation of subfraction F7 against the MCF-7 cells line exhibited IC_{50} values of 15.154 µg/mL.

Figure 1. The profile of *A. nakamurai* sample and its spicule.

Table 1. The fractions activity against breast cancer cells MDA-MB-231.

No.	Solvent extraction	IC_{50} (µg/mL)
1	EtOAc	13.33
2	*n*-Butanol	1,430.44
3	Water	756.34

This IC_{50} value was stronger than the positive control cisplatin with IC_{50} 81.052 µg/mL.

The profiling of the proliferation test described that fraction 7 inhibited the MDA-MB-231 cells' growth until the lowest concentration at 7.81 µg/mL, with the inhibition of 45.389% (Figure 2). Even though this value was higher than IC50 doxorubicin (0.58 µg/mL) [19], this subfraction could be further investigated.

To know the cytotoxic activity against normal cells, all of the subfractions were evaluated against the human embryonic kidney HEK-293 cell line. The result is shown in Table 3.

Data in Table 3 showed that fraction 7 was cytotoxic against HEK-293 with the % viability value at the concentration of 25 µg/mL was 6% ± 1.8%. The noncytotoxic effect of the HEK-293 cells line is indicated by 90%–95% cell viability at the concentration of 100 µg/mL sample [20].

The cytotoxicity of subfraction F7 was abroad to the breast cancer MDA-MB-231 and MCF-7 as well as normal human embryonic kidney cell lines HEK-293. Further study to enhance the toxicity is recommended to find the selective cytotoxic against breast cancer cell lines such as MDA-MB-231 and MCF-7 cells.

Table 2. The result of EtoAC fractionation and the cytotoxic assay against MDA-MB-231 and MCF-7 cell lines.

Sample	Solvents	Weight (mg)	IC_{50} (µg/mL)	
			MDA-MB-231	MCF-7
F1	*n*-Hexane	5.7	>1,000	nd
F2	Hexane/CH_2Cl_2 (3/1)	2.41	>1,000	nd
F3	Hexane/CH_2Cl_2 (1/3)	42.38	914.208	nd
F4	CH_2Cl_2	19.49	>1,000	nd
F5	EtOAc/CH_2Cl_2 (1/1)	91.44	>1,000	nd
F6	EtOAc	20.63	>1,000	nd
F7	MeOH	1,168.6	10.677	15.154
Cisplatin	-	-	23.56	81.052

nd : not determined.

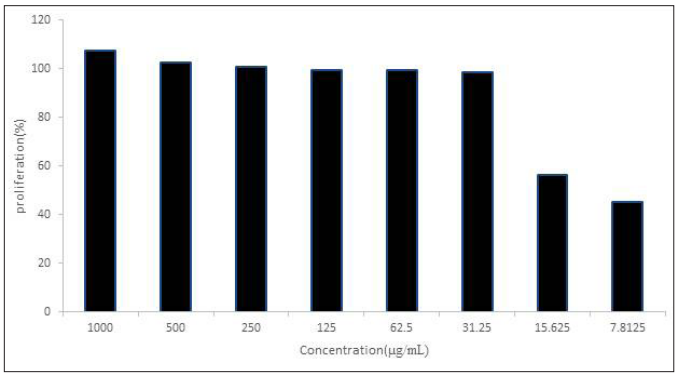

Figure 2. Growth-inhibition of MDA-MB-231 treated with fraction 7 in several concentration.

LC-MS/MS analysis

The LC-MS/MS data of EtOAc fraction and subfraction F7 are described in Table 4.

The active compound detected in EtOAc fraction at a retention time of 8.60 with an exact mass of 436.309 Da was predicted to be similar to the halogenated pyrrole compound midpacamide $C_{13}H_{16}Br_2N_4O_3$ with a calculated molecular weight of 436.300 Da [6]. The electron spray ionization mass spectroscopy (ESIMS) data showed that midpacamide had the ion-molecule at *m/z* 437.309 $[M+H]^+$. Previous research reported that midpacamide, with a molecular weight of 436.1 and an exact mass of 435.956 Da, was isolated from *A. nakamurai* collected from Menjangan Island, Bali. Midpacamide was found to have moderate activity against L5178Y, with IC_{50} values of 10 µg/mL [6]. Another peak of EtOAc fraction was detected at retention 9.45 minutes and was identified as agelasin-D with molecular weight of 421.31 Da. The unknown compound was also detected at a retention time of 1.6643 minutes. The separation of EtOAc fraction generated active subfraction F7. The profiling of compounds using LC-MS/MS is described in Figure 3.

There were two intense peaks appearing in the subfraction F7 at retention times of 1.2 and 0.46 minutes. The detailed analysis of ESIMS, at the retention time of 1.2 minutes, elicited molecular ion at *m/z* 422.3274 Da $[M + H]^+$, which was later identified as agelasin-D. The previous researcher also reported that agelasine D has been found in *A. nakamurai* [17] and *Agelas mauritania* [8]. The agelasines group was the chemical marker of *Agelas* sponges that was found in several species of *Agelas* and microbes associated with this sponge, including agelasin A-I isolated from *A. nakamurai* and Agelasin B found in the fungus *Agelas cf. mauritiana* [6,21–23]. In addition, agelasine D was reported as an anti-dormant-mycobacterial [21].

The other compound at retention 0.46 detected in subfraction 7 was ageloxime-D. Previous research reported that ageloxime-D is active against L5178Y mouse lymphoma cells [6] and antimicrobial [8]. The oxime group in ageloxime D was reported to increase the biofilm activity but reduce the toxicity to bacteria [24].

Ageloxime-D in the LC-MS chromatogram peak showed a double peak because of the oxime group's tautomerism. The tautomerism of the oxime group in ageloxime-D also gives the splitting peak in the UV spectrum and duplication resonance in the nuclear magnetic resonance spectrum and appearing ion molecule *m/z* at 440.3 and 422.3 [6].

Table 3. The result of the cytotoxic assay against the HEK-293 cell line.

Fractions	% cells viability (at 25 µg/mL)
F1	na
F2	88 ± 7.1
F3	56 ± 2.4
F4	78 ± 4.3
F5	85 ± 2.0
F6	61 ± 3.5
F7	6 ± 1.8

Table 4. LC-MS data of EtOAc fraction (a) and subfraction F7 (b).

a)

Retention time (minute)	Neutral mass (Da)	Neutral mass (Da) (reference)	Prediction compound	Source/references
1.66	127.086580		Unknown	-
8.61	436.30936	436.10	$C_{13}H_{16}Br_2N_4O_3$,midpacamide	*Agelas nakamurai*, *Agelas mauritiana*/[6]
9.49	421.32659	421.31921	-(-) Agelasin-D ($C_{26}H_{41}N_5$)	*Agelas nakamurai*, *Agelas mauritiana* [6]
10.09	437.31546		Unknown	-

b)

Retention time (minute)	Ion molecule $[M + H]^+$	Theoretical mass $[M + H]^+$	Prediction compound	Source/References
1.2	422.3274	422.32748	Agelasin-D ($C_{26}H_{40}N_5$)	Instrument library-
0.46	440.3382		Ageloximed-D	[6]

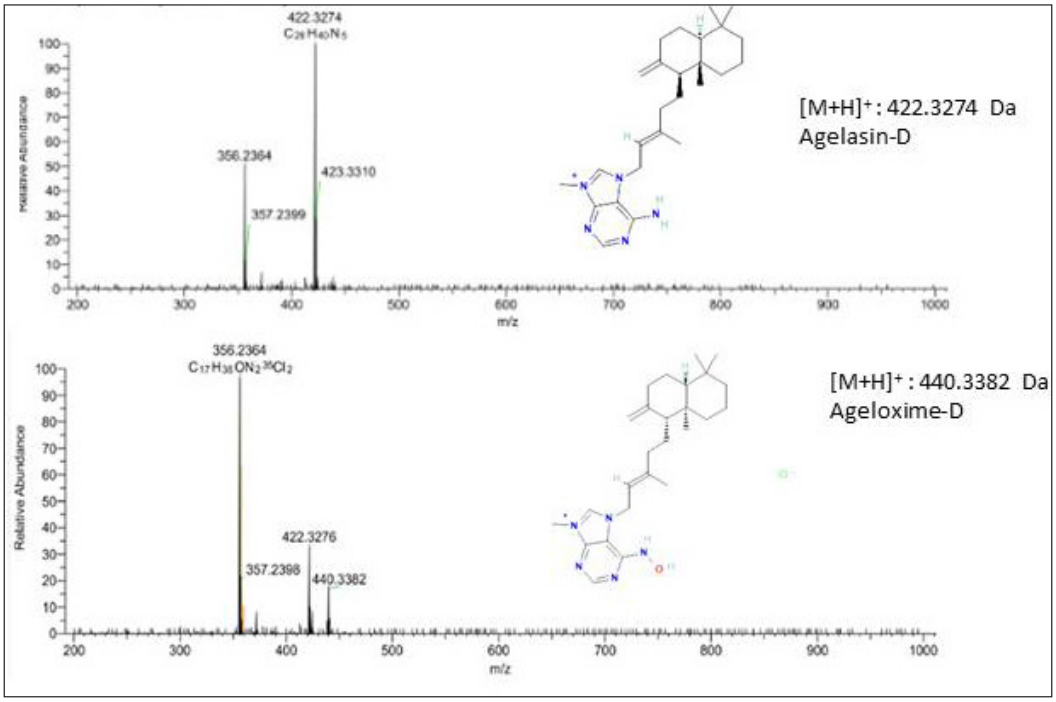

Figure 3. The profile of MS/MS chromatogram of active subfraction F7.

CONCLUSION

Considering the data obtained in this study, the *A. nakamurai* was the potential source of compounds that were strongly active against MDA-MB-231 (IC_{50}: 10.677 µg/mL) and MCF-7 (IC_{50}:15.154 µg/mL) cell lines. Active subfraction F7 contained the agelasin-D and ageloximed-D. Even though the subfraction F7 showed cytotoxicity against HEK-293, further structural optimization was needed to decrease cytotoxicity against normal cell lines and increase in the targeted cancer cells.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took Fart in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author

as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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