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Research Article

Cytotoxicity and Antiproliferative Activity of Indonesian Red Algae Acanthophora muscoides Crude Ethanol Extracts

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Abstract

Background and Objective: Red macroalgae Acanthophora muscoides is widely distributed in Indonesian oceans. However, information regarding their bioactive properties and pharmaceutical potentials are largely unexplored. This study evaluated the cytotoxicity and antiproliferative activity of ethanol extracts of Acanthophora muscoides and compare with common species Acanthophora spicifera collected from Indonesia. **Materials and Methods:** Cytotoxicity and antiproliferative activity of A. spicifera (AS-EtOH) and A. muscoides (AM-EtOH) crude extracts were evaluated with Lactate dehidrogenase (LDH) and Trypan blue

exclusion assay from 6.25-200 µg mL^{-1} concentration in Human cervical cancer (HeLa) cells for 72 h. Viability staining with nucleus staining Hoechst33342/PI was conducted to investigate apoptotic activity. Potential cell death mechanisms of treatments were determined by DNA fragmentation assay. **Results:** AM-EtOH demonstrated high cytotoxicity in HeLa cells with EC_{50} of 180±14 µg mL^{-1}. However, AS-EtOH EC_{50} values could not be determined in this study. Antiproliferative activity was significant in AM-EtOH treated cells as cell density was suppressed to 29×10^4 cells mL^{-1} compared to AS-EtOH (70×10^4 cells mL^{-1}) and untreated cells (78×10^4 cells mL^{-1}). Finally, DNA fragmentation with a ladder pattern characteristic of apoptosis was observed in AM-EtOH treated HeLa cells. Cells treated with AS-EtOH were seen to show significant cytotoxic effects towards cell viability, but not to the point of death through apoptosis. **Conclusion:** Current results show AM-EtOH possesses more promising bioactive properties, cytotoxic and antiproliferative activity compared to AS-EtOH. Further advanced studies would be reasonable for AM-EtOH potential uses in pharmaceutical industries.

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INTRODUCTION

The number of cancer incidences are increasing and becoming a cause of death worldwide. Due to the increases in lifestyles associated with economic development, the cancer prevalence has spread not only in developed countries, but also **developing countries** (http://www.scialert.net/asci/result.php?searchin=Keywords&cat=&ascicat=ALL&Submit=Search&keyword=developing+countries), such as Indonesia[1]. In the last decades, the search and development of new drugs have increased and nature became a relevant resource for the discovery of anticancer compounds. Today, approximately 307 natural products and 15 natural product derivatives from plant, bacteria, fungi and marine sources had been approved by the Food and Drug Administration (FDA) or 21% of all approved New Chemical Entities (NCEs)[2].

Marine organisms have already proved to be a rich source of bioactive compounds and as a result, their exploration for pharmacological purposes has increased greatly in recent years. Macroalgae or seaweeds are one example of marine organisms which has attracted the attention of the pharmaceutical industry. As a mechanism to protect themselves against other organisms in their environment, macroalgae produce a wide variety of bioactive metabolites as potential source of novel compounds for various purposes[3].

Hence, macroalgae has the ability to produce secondary metabolites with various pharmacological activities such as **cytotoxic activity** (http://www.scialert.net/asci/result.php?searchin=Keywords&cat=&ascicat=ALL&Submit=Search&keyword=cytotoxic+activity), antiproliferative, antimicrobial, cytoprotective, anticoagulant and antioxidant activities[4-11].

Although highly distributed in Indonesian waters, regardless no traditional and biological studies have locally been reported on this Acanthophora macroalgae. In Indonesia the study are more focusing on the ecological data[12]. Indonesian tropical warm waters, is well known as a suitable place for macroalgae

growth. Several studies have revealed biological activities in AS-EtOH in cancer cell lines[13,14]. However, there remains little and almost no established information regarding AM-EtOH anticancer bioactive potentials. Other compounds will be present in crude extracts and the effects of these impurities, either synergistic or antagonistic are unknown. Hence, preliminary screening is necessary to understand the synergistic interaction or multi-factorial effects in crude extracts for determination of pharmaceutical potentials. The present study was therefore to evaluate potential antiproliferative activities of AM-EtOH crude extract and also compare it with common Acanthophora species, AS-EtOHon an **in vitro** (http://www.scialert.net/asci/result.php?searchin=Keywords&cat=&ascicat=ALL&Submit=Search&keyword=in+vitro) carcinoma model of HeLa cells. Current result would provide information regarding potential development of red macroalgae AM-EtOH from Indonesia in medicinal and pharmaceutical industries.

MATERIALS AND METHODS

Sample collection and extraction: Red macroalgae Acanthophora were collected from North West Lombok coastal area (8°24’11.7396"S, 116°4’1.9056"E), West Nusa Tenggara province, Indonesia in the end of 2017. Macroalgae specimens were identified according to electronic algae database[15,16]. The seaweed samples were washed with freshwater to remove adhering debris. The collected samples were then dried and powdered. Powder samples were mixed with absolute ethanol solvent with 5×volume of sample weight (w/v). Suspensions were then macerated by incubation in room temperature for 48 h. Every 24 h, suspensions were filtered with Whatman number 1 filter papers. Filtrates were evaporated with rotary evaporators until concentrated ethanol extracts were obtained. These filtrates were then used for seaweed ethanol extracts.

Chemicals and reagents: The 2-(4-Iodophenyl)-3-(4 -nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1), 1-methoxyl-5-methylphenazinium methylsulfate (1-Met-PMS) were purchased from Dojindo Laboratories, (Kumamoto, Japan), nicotinamide adenine dinucleotide (NAD) was obtained from Oriental Yeast. Co. Ltd., (Tokyo, Japan). General reagents including lithium lactate were purchased from Wako Pure Chemicals (Osaka, Japan).

Preparation of macroalgae extract for analysis: Powdered seaweed (50 g) was extracted successively using Soxhlet extractor sequentially with different solvents of increasing polarity of ethanol until the extract was clear[17]. The resulting pasty extracts were stored in a refrigerator at 4°C for further use. The extraction was centrifuged at 15,000×g for 10 min. The supernatant was collected and dried and stored at 4°C until future use. The dried macroalgae extract was resolved in dimethyl sulfoxide (DMSO) and centrifuged at 15,000×g for 15 min, then obtained clear macroalgae extract sample.

GC-MS analysis: Gas Chromatography-mass Spectrometry (GC-MS) analysis of the ethanol extract of AS-EtOH and AM-EtOH were performed using a Perkin-Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a gas chromatograph interfaced to a mass spectrometer (GC-MS) equipped with a Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused a capillary column (30×0.25 µm ID×0.25 µm df). For GC-MS detection, an electron ionization system was operated in electron impact mode with anionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 mL min^{-1} and an injection volume of 2 µL was employed (a split ratio of 10:1). The injector temperature was maintained at 250°C, the ion-source temperature was 200°C, the oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C min^{-1} to 200°C, then 5°C min^{-1} to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV, a scan interval of 0.5 sec and fragments from 45-450 Da. The solvent delay was 0-2 min and the total GC/MS running time was 36 min. The relative

percentage amount of each component was calculated by comparing its average peak area to the total areas. The mass-detector used in this analysis was Turbo-Mass gold-perkin-elmer and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver-5.2.

Cell culture: Human cervical cancer cell line (HeLa) cells were routinely cultivated in Dulbecco's Modified Eagle Medium (DMEM, Wako) supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 humidified incubator. For all experiments, HeLa cells were plated in DMEM, incubated overnight and incubated in DMEM supplemented with specific concentrations of macroalgae extracts. Cell images were obtained from phase-contrast microscopy by BZ-9000 microscope (Keyence, Osaka, Japan).

Cytotoxicity assay and cell proliferation: Cytotoxicity of AS-EtOH and AM-EtOH were estimated by lactate dehydrogenase (LDH) assay and cell proliferation was measured by Trypan blue dye exclusion assay[18,19]. Cells were cultured in 96-well culture plate over-night, then the culture mediums were discarded and changed with new medium containing several concentrations (6.25-200 μg mL^{-1}) of AM-EtOH and AS-EtOH. After 24 h incubation, a portion of the culture medium (±100 μL) was transferred to another 96-well plate, which contained 2.5 mM NAD, 50 mM lithium lactate, 100 mMTris (pH 8.0), 0.04 mM 1-Met-PMS and 1 mM WST-1. Colouring change depended on LDH activity was measured by absorbance at 450 nm. Maximum absorbance (A_{450max}) was obtained from completely dead cells by addition of 1% Triton X-100 in the cell culture and minimum absorbance (A_{450min}) was obtained from the control cell culture. Cytotoxicity was calculated by the following equation:

$$\text{Cytotoxicity (\%)} = \frac{A_{450\,\text{treatment}} - A_{450\,\text{min}}}{A_{450\,\text{max}} - A_{450\,\text{min}}} \times 100$$

Cell proliferation was measured by counting cell number under microscopic observation. The cell suspension was mixed with equal volume of 0.4% Trypan blue dye. Viability was indicated as the ratio of live cells to total cells.

Hoechst33342-PI viability staining: Cells were incubated with macroalgae extracts for 72 h prior for early apoptotic detection with Hoechst33342/PI viability staining[20]. Treated cells were stained 2 μL volume of Hoechst33342 and incubated for 15 min in CO_2 and 37°C incubator. Followed by staining with Propidium Iodide (PI) with the same volume and analyzed using fluorescence microscopy and ImageJ software.

DNA fragmentation: The cells were incubated with 100 μg mL^{-1} concentration of AS-EtOH and AM-EtOH for 3 days. The treated cells were collected by trypsinization. DNA was extracted once, with an equal volume of phenol: Chloroform: Isoamyl alcohol (25:24:1) and once with chloroform: Isoamyl alcohol (24:1). The DNA was precipitated with a two-thirds volume of cold isopropanol followed by centrifugation. The DNA pellet was washed once in 70% ethanol and resuspended in deionized water containing 0.1 mg mL^{-1}. DNA was analyzed by 1.5% agarose gel electrophoresis. Staurosporine (STS) was used as positive control[21].

Statistical analysis: Student's t-test was used to analyze intergroup differences. Experiments were repeated at least three times and data are represented as the Mean±A. p-value of less than 0.05 was considered to be statistically significant.

RESULTS

Bioactive compounds present in macroalgae AS-EtOH and AM-EtOH extracts: The bioactive compounds present in crude extracts obtained from AM-EtOH and AS-EtOH are shown in Fig. 1. Their identification and characterization were based on their retention order in a HP-5MS coloumn. The retention time, molecular formula and the amount of these bioactive compounds were also presented (Table 1). Based on abundance, top three major compounds present in AS crude extract were palmitic acid (9.56%), octadecanoic acid (7.55%) and pentadecanoic acid (4.53%). Whereas, AM crude extract shown more diverse presence of main compounds, such as 2,3,5-Tribomo-N-methylindole (16.24%), octadecanoic acid (23.67%) and palmitic acid (9.09%).

Cytotoxicity and antiproliferative assay: Cytotoxicity and antiproliferative effects of red seaweed AS-EtOH and AM-EtOH extracts were investigated via Lactate dehydrogenase (LDH) assay against HeLa human cervical cancer cell line. Cytotoxic activity of Acantohphora species ethanol extracts increased in a dose-dependent manner (Fig. 2).

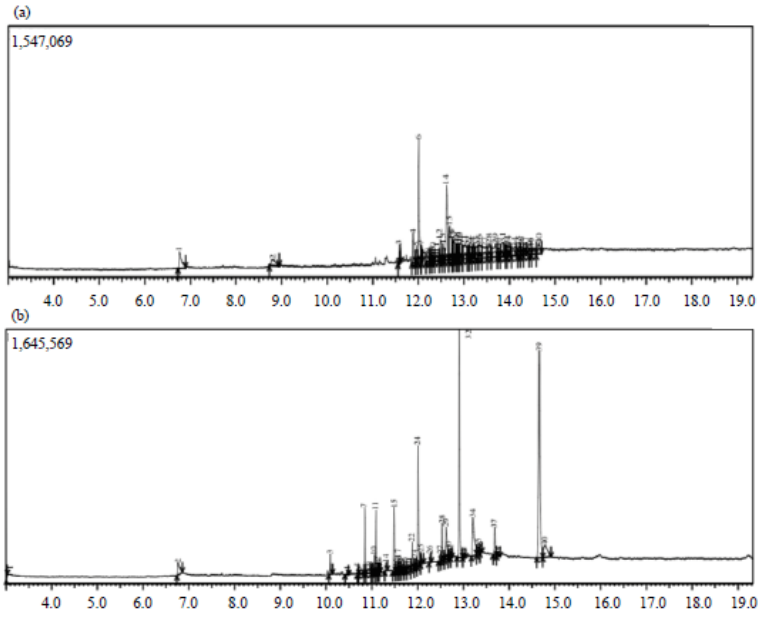

Fig. 1(a-b): GC-MS spectrum of (a) AS-EtOH and (b) AM-EtOH

Table 1: GC-MS data of AS-EtOH and AM-EtOH ethanol extracts

Samples	Name of compound	RT	Peak	Area (%)	Molecular form	Molecular weight (g mol^{-1})
AS-EtOH	Hexanal	11.908	40	1.750	$C_6H_{12}O$	100.161
	Palmitic acid	12.617	60	9.560	$C_{16}H_{32}O_2$	256.430
	1-ethyl-4(1H)-Quinolinone	12.521	12	2.590	$C_{11}H_{11}NO$	173.215
	Octadecanoic acid	12.811	14	7.550	$C_{18}H_{36}O_2$	284.484
	Pentadecanoic acid	12.862	15	4.530	$C_{15}H_{30}O_2$	210.405
AM-EtOH	1,1,3,3-Tetramethylbutyl isocyanide	3.0250	10	0.410	$C_9H_{17}N$	139.242
	Acetal	6.7640	20	2.030	$C_6H_{14}O_2$	118.176
	Calarene	10.093	30	1.330	$C_{15}H_{24}$	204.357
	β-copaen-4-α-ol	10.846	70	3.470	$C_{15}H_{24}O$	220.356
	Heptadecane	11.070	10	1.470	$C_{17}H_{36}$	240.475
	1-Naphthalenamine, 4-bromo	11.089	11	3.490	$C_{10}H_8BrN$	222.085
	Valerenal	11.484	15	4.410	$C_{15}H_{22}O$	218.340
	Palmitic acid	12.010	24	9.090	$C_{16}H_{32}O_2$	256.430
	Veridiflorol	10.461	25	0.420	$C_{20}H_{32}O_7$	399.484
	Phytol isomer	12.531	28	2.730	$C_{20}H_{40}O$	296.539
	Heptadecene-(8)-carbonic acid	12.620	29	4.130	$C_{17}H_{32}O_2$	268.430
	2,3,5-Tribomo-N-methylindole	12.910	32	16.24	$C_9H_6Br_3N$	367.866
	Oleic acid	13.207	34	6.100	$C_{18}H_{34}O_2$	282.468
	Hexanedioic acid, dioctyl ester	13.688	37	2.470	$C_6H_{10}O_4$	146.142
	Octadecanoic acid	14.660	39	23.67	$C_{18}H_{36}O_2$	284.484

RT: Retention Time

After 72 h, the EC_{50} value of AM-EtOH crude extract was 180±14 µg mL^{-1}. Whereas, EC_{50} value of AS-EtOH crude extract could not be determined in this study. AS-EtOH and AM-EtOH anti-proliferative effects were determined with cell count by trypan blue exclusion assay. Untreated cells showed high cell density with total cell count approximately 78×10^4 cells mL^{-1} in 72 h (Fig. 3). Treated with AM-EtOH extracts resulted in suppressed cell density (29×10^4 cells mL^{-1}). In contrast, cell density in As-EtOH treated cells (70×10^4 cells mL^{-1}) demonstrated no antiproliferative activity. Acanthophora extracts of concentration over 200 µg mL^{-1} resulted in some debris which appeared in the culture medium, therefore, it could not perform higher concentration in this study.

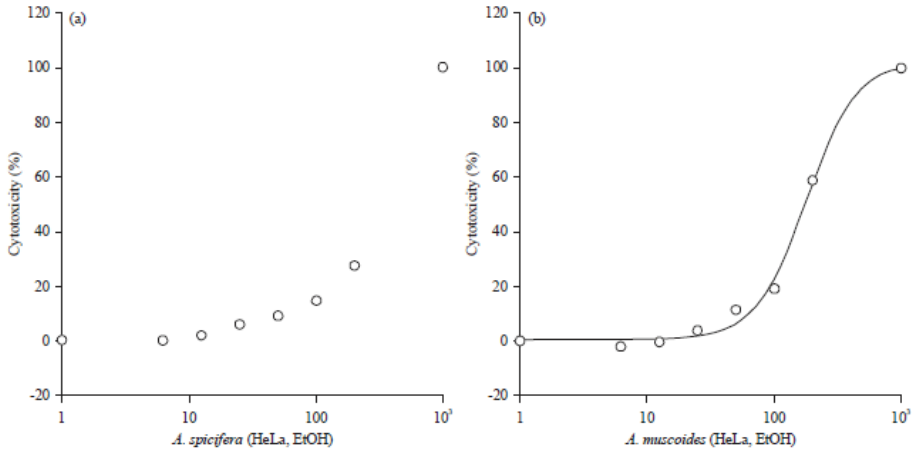

Fig. 2(a-b): Cytotoxic assay of HeLa incubated cells with (a) AS-EtOH and (b) AM-EtOH for 72 h analyzed with LDH assay. Graph fitting for dose response analyses were conducted with Kaleidagraph software

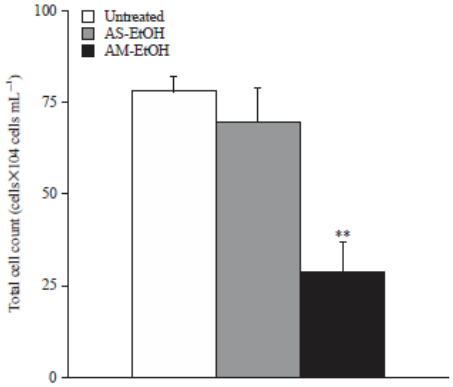

Fig. 3: Anti-proliferative assay of HeLa incubated cells with AS-EtOH and AM-EtOH for 72 h. Total cell count was conducted with Trypan blue exclusion assay

*t-test ($p<0.05$) significantly different, **($p<0.001$) highly significant compared to control

Hoechst3342-PI viability staining: Morphology observation under fluorescence microscope shown that untreated HeLa cells were homogeneously distributed, exhibit an elongated shape (Fig. 4). Nucleuses staining with Hoechst33342/PI stained live/dead cells with blue fluorescence dye Hoechst33342 and dead cells were stained red fluorescence dye Propidium iodide (PI). These nucleic dyes would bind to specific regions in DNA, so stained particles are assumed to be cells. Dead cell (%) was seen to significantly increase in AS-EtOH and AM-EtOH treated cells (Fig. 5). Overall, both extract treatments induce changes in cell morphology. These alterations induced by the treatment included reduction of cell size, cell shrinkage and the formation of blebs on cell surface resulted in the generation of apoptotic bodies.

DNA fragmentation: DNA fragmentation assay was used to determine the action of AS-EtOH and AM-EtOH extracts was associated with apoptosis or not. As shown in previous results, AM-EtOH extract was strongly effective in inhibiting HeLa cell proliferation. The ladder pattern was observed for a maximum of 3 days because after that all cells died. DNA fragmentation was clearly observable in AM-EtOH treated HeLa cells (Fig. 6). Cells treated with AS-EtOH were seen to show inhibited cell growth, but not to the point of death through apoptosis.

DISCUSSION

Macroalgae or seaweed has attracted an emerging interest in the biomedical area, mainly to their content of variable compounds with therapeutic value. Several studies have reported macroalgae to contain various compounds with various biological activities such as sulfated polysaccharides, polyphenols and pholorotannins[22-24]. In this study, the antitumor potential of Acanthophora species that are abundant in Indonesia were evaluated. Among the tested algae were Acanthophora spicifera (AS-EtOH) and Acanthophora muscoides (AM-EtOH) which are most common species of Acanthophora in Indonesia.

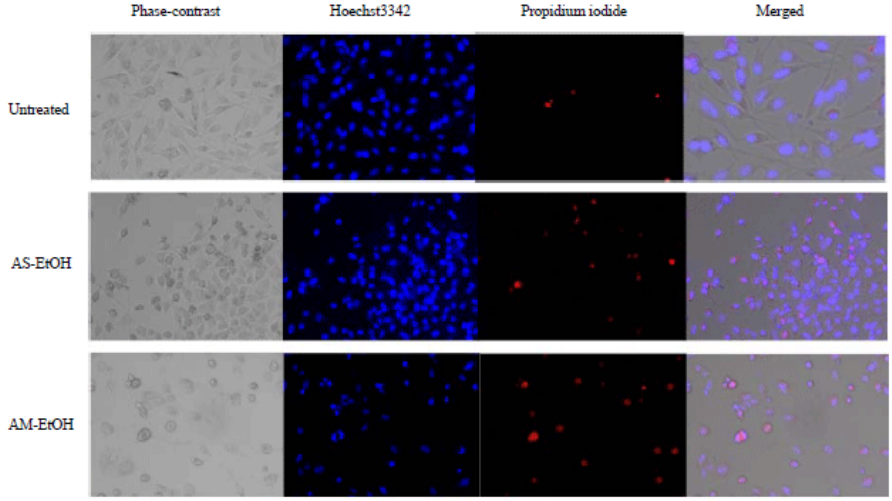

Fig. 4: Fluorescence microscopy observation of HeLa cells incubated with 20 μg mL^{-1} of AS-EtOH and AM-EtOH extract for 72 h. Blue cells stained with Hoechst 3342 incubated live/dead cells. Red cells stained with propidium lodide indicate dead cells

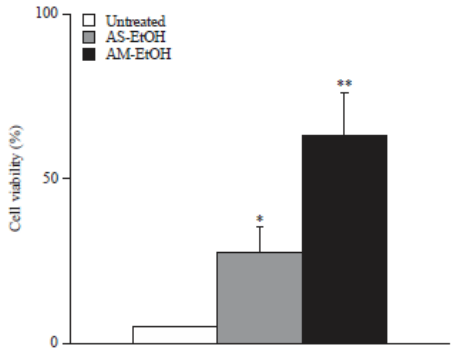

Fig. 5: Dead cell percentage AS-EtOH and AM-EtOH 200 μg mL^{-1} treated HeLa cells for 72 h. Viability percentages were calculated with ImageJ software.

*t-test ($p<0.001$) highly significant compared to control

Despite the high degree of availability of these species, there remains little information regarding its bioactive properties and therapeutic potentials. Our current results showed that AM-EtOH extract presented the highest potential in both cytotoxic and antiproliferative studies.

The activity evidenced by AS-EtOH and AM-EtOH extracts can be associated with the presence of several bioactive compounds shown in chromatographic profiles (Fig. 1). Major compounds present in AS crude extract are Pentadecanoic acid (10.38%), Octadecanoic acid (7.55%) and palmitic acid (9.56%).

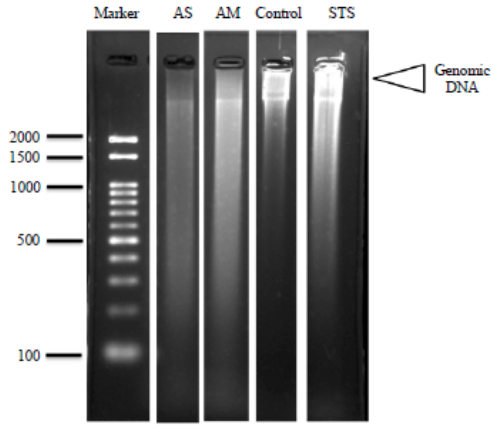

Fig. 6: DNA ladder assay for detection of apoptosis in HeLa cells treated with seaweed extracts. HeLa cells were cultured in DMEM with 10% FBS and 200 µg mL^{-1} extracts. Apoptotic cells were induced by 4 µM staurosporine (STS) for 4 h incubation as positive control. 5 µg DNA of each sample was electrophoresed on a 2% agarose gel and the gel was stained by ethidium bromide

Whereas, AM-EtOH crude extract shown more promising compounds, such as 2,3,5-Tribomo-N-methylindole (16.24%), Octadecanoic acid (23.67%) and Palmitic acid (9.09%). Compound 2,3,5-Tribomo-N-methylindole is a marine indol alkaloid that has been well reported to demonstrate antitumor therapeutic activity[25,26]. Palmitic acid and Octadecanoic acid also showed anticancer potential with apoptosis inducing activity in human cancer cell lines[27,28]. Synergistic interaction or multi factorial effects between compounds could be present in plant extracts. Hence, pure compounds that are isolated form plants may not have the same degree of activity as the unrefined crude extract at comparable concentrations or dose of the active component[29]. Due to the limited information regarding these Acanthophora sp., in this study we investigated the therapeutic potentials of crude extracts as basic information for development of this species towards potential therapeutic applications.

The cytotoxic effects of AS-EtOH and AM-EtOH were determined with LDH assay. Membrane leakage as confirmed by LDH assay indication cell injury which potentially induces DNA damage and consequently cell death[30,31]. Lactate dehidrogenase is a soluble cytosolic enzyme, which is released into the extracellular medium due to membrane damage consequently leads to cell death. Thus the content of Acanthophora extracts induced cytotoxicity was determined by measuring the activity of LDH in the supernatant as indirect method[27]. In this study, the LDH activity in the medium was significantly higher in AM-EtOH treatment with EC_{50} of 180±14 µg mL^{-1} (Fig. 2). In contrast, AS-EtOH cytotoxicity could not be determined in this study. Ethanol dissolves a substantial number of substances due to its polar and non-polar nature. It has a polar hydroxyl group that could form H-bonds, whilst there is an alkyl non-polar part that could form dispersion forces with grease and other non-polar substances[32]. Hence, most of the targeted bioactive compounds are to mostly dissolve in ethanol. The antiproliferative activity AS-EtOH and AM-EtOH were measured with Trypan blue dye exclusion assay[33]. Significant decrease in cell density was clearly observable in AM-EtOH treated cells (29×10^4 cells mL^{-1}) compared to AS-EtOH (70×10^4 cells mL^{-1}) and untreated cells (78×10^4 cells mL^{-1}) (Fig. 3). Potential bioactive compounds with antitumor potential showed higher concentrations in AM-EtOH such as palmitic acid, octadecanoic acid and alkaloid 2,3,5-Tribomo-N -methylindole. Literature reports showed that Palmitic acid and Octadecanoic acid could inhibit growth in cancer cells[27,34]. Additionally, AM-EtOH showed existence of a marine indole alkaloid 2,3,5-Tribomo-N-methylindole. Marine natural alkaloids are evidenced to exhibit various pharmaceutical properties[35,36].

Observation of morphological characteristics remains a key parameter for early determine of apoptosis[37]. AS-EtOH and AM-EtOH crude extracts treated HeLa cells were stained with Hoechst33342 and Propidium Iodide (PI). Hoechst dyes interact with nucleotides to emit fluorescence. These dyes are positively charged under physiological conditions and can pass through viable membranes. In this study, Hoechst33342 blue fluorescence dye is used to determine live and dead cells. Propidium Iodide molecules intercalate inside the DNA double helix and impermeable through the cell membrane of viable cells[38,39]. Exposure of cells to Acanthophora extracts after 72 h results in altered morphological changes (Fig. 4). Previous result showed AS-EtOH to have little effect on cell proliferation. However, nucleic staining revealed that AS-EtOH treatment possesses cytotoxic effect which significantly decreases dead cell percentage (Fig. 5). In contrast, AM-EtOH demonstrated strong cytotoxic and antiproliferative activity. This was evidenced by morphological changes such as cell shrinkage and bleb formation in AM-EtOH treated cells. Although several studies have reported anticancer potential of AS-EtOH against different types of cell lines[13,14,40] no report has described the mechanism of AS-EtOH cytotoxicity. Furthermore, to our knowledge, this is the first report to evaluate pharmaceutical potentials of red macroalgae of Acanthophora muscoides.

One of the biochemical hallmarks of apoptosis is degradation of DNA by endogenous DNAses, which cut inter-nucleosomal regions into DNA fragments. The DNA fragmentation forms a ladder pattern that can be used to determine cell death mechanism[41,42]. This phenomenon can be generally detected by agarose gel electrophoresis, as shown in Fig. 6. Here, our results showed that the DNA ladders of HeLa cells treated with AS-EtOH and AM-EtOH were observed within maximum of 3 days, because after that all cells died. Apoptotic activity was detected in AM-EtOH treated HeLa cells which showed DNA fragmentation similar to positive control Staurosporine (STS). In contrast, fragmented DNA was not clear in AS-EtOH treated cells. Therefore, apoptotic activity could only be confirmed in AM-EtOH treated HeLa cells.

These results indicated the presence of compounds with high potency. Moreover, the extracts here tested are a complex mixture of compounds and the portion of active compounds may be very low. These data supported the view that Acanthophora muscoides can be an interesting source of molecules with antitumor potential.

CONCLUSION

AM-EtOH from red macroalgae Acanthophora muscoides exhibited significant cytotoxic and antiproliferative activity against HeLa cells compared to AS-EtOH. The cytotoxic effect of AM-EtOH appears to have induced apoptosis in HeLA cells. This study is one of many steps required in order to obtain a therapeutic molecule. Further purification to obtain a potentially active and pure compound will be undertaken in the future. Nevertheless, to our knowledge this is the first therapeutic potential screening on Acanthophora macroalgae from the Lombok coast Indonesia revealing antitumor potential that opens a new opportunity window for cancer prevention.

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SIGNIFICANCE STATEMENT

Red Macroalgae Acanthophora has high abundance in Indonesian coastal waters. Most abundant species are Acanthophora spicifera (AS) and Acanthophora muscoides (AM). Several studies have evidenced biological activities of AS. However, most studies only describe ecological aspects of Indonesian Acanthophora. Furthermore, there remains no information regarding pharmaceutical properties of AM. The various parameters studied are bioactive compound analyses in ethanol crude extracts, cytotoxicity and

antiproliferative analysis, microscopy, cellular and molecular analysis. Our findings suggest strong antiproliferative activity of AM. Further purification to obtain a potentially active and pure compound will be undertaken in the future for potential development of AM in cancer prevention.

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