

# Effectiveness of Methanol Solvent Extraction for Red Macroalgae *Acanthophora spicifera* Antitumor Activity

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## ABSTRACT

**Introduction:** Red macroalgae *Acanthophora spicifera* is commonly found in most parts of Indonesia coastal areas. However, information regarding their potential pharmaceutical uses remains largely unexplored. This study evaluates the antiproliferative activity of *Acanthophora spicifera* collected from Indonesian extracted with different solvents of ethanol and methanol.

**Methods:** Cytotoxicity and antiproliferative activity of *A. spicifera* crude ethanol extract (AS-EtOH) and *A. spicifera* crude methanol extract (AM-MetOH) were evaluated with Lactate dehydrogenase (LDH) and Trypan blue exclusion assay in Human cervical cancer (HeLa) cells for 72h. Concentration of extracts treated in HeLa cells were 6.25 to 200  $\mu\text{g/mL}$ . Viability staining with fluorescence stain Hoechst33342/PI was conducted to investigate apoptotic activity. Potential apoptotic activity of treatments was confirmed by DNA fragmentation assay.

**Results:** AS-MetOH demonstrated high cytotoxicity in HeLa cells with  $\text{IC}_{50}$  of  $127.3 \pm 33.13 \mu\text{g/mL}$ . However, AS-EtOH  $\text{IC}_{50}$  values could not be determined in this study. Antiproliferative activity was highly significant in AS-MetOH treated cells as cell density was suppressed to  $20 \times 10^4$  cells/mL compared to AS-EtOH ( $73 \times 10^4$  cells/mL) and untreated cells ( $83 \times 10^4$  cells/mL). Finally, apoptotic activity could be determined in AS-MetOH treated cells with increase PI fluorescence emitting cells and DNA fragmentation. On the other hand, apoptotic activity was not observable in AS-EtOH treated cells based on fluorescence viability staining and DNA fragmentation assay. **Conclusion:** Current results show methanol solvent as an effective solvent to promote potential pharmaceutical properties of macroalgae *A. spicifera*. Further advanced studies in a compound level from *A. spicifera* methanol fraction would be reasonable for development of macroalgae based anticancer agent.

**Key words:** *Acanthophora*, Antiproliferative, Cytotoxicity, Indonesia, Macroalgae.

## INTRODUCTION

Cancer is a leading cause of death worldwide after cardiovascular diseases. Although various anticancer agents have been discovered and are in use currently, cancer treatment remains a large health challenge.<sup>1</sup> Common cancer treatments such as chemotherapy are well known to result in adverse side effects.<sup>2</sup> The costs of such treatments are unaffordable for most people particularly in developing countries such as Indonesia. Hence, research on discovery of alternative and complementary anticancer drugs from natural products continues to increase in rapid pace. Indonesia is a country with high diversity, especially in parts of its oceans which most of the bioresources remain largely unexplored. Recent studies have evidenced medicinal potentials of marine organisms as potential source of anticancer agents.<sup>3</sup> Macroalgae or seaweeds are one example of marine organisms which provide promising anticancer activity.<sup>4,5</sup> An extract from the brown seaweed *Sargassum thunbergii* has shown antitumor activity and inhibition of tumour metastasis in the rat mammary adeno carcinoma cell.<sup>6</sup> Some studies

recently also evaluated the effect of a brown seaweed (*Sargassum muticum*) methanol extract on the proliferation of MCF-7 and MDA-MB-231 breast cancer cells by conducting morphological assessments of apoptosis, caspase assays and chick Chorioallantoic Membrane (CAM) assays.<sup>7</sup> Indonesia is well known for its optimal condition for macroalgae growth.<sup>8</sup> Despite its abundance, there remains little information regarding potential utilizations of macroalgae in pharmaceutical industries.

One common red macroalgae species which is commonly found in Indonesian coastal areas is *Acanthophora spicifera*. The alcoholic extract of the red algae *Acanthophora spicifera* exhibit antitumor activity on Ehrlich's ascites carcinoma cells developed in mice at a dose of 20 mg/kg, comparable to the standard drug 5-fluorouracil.<sup>9</sup> This is evidenced by increase in the mean survival time, decrease in tumor volume and viable cell count. Furthermore, the seaweeds *Acanthophora spicifera*, *Ulva reticulata*, *Gracilaria foliifera* and *Padina boergesenii* of the

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Gulf of Manna region are evidenced to demonstrate cytotoxic activity in their alcoholic extracts.<sup>3</sup> However, the cytotoxic effect of *A. spicifera* reported in previous study is considered as low cytotoxic activity ( $IC_{50} \geq 500 \mu\text{g/mL}$ ). Hence, in our study we evaluate the effectiveness of methanol solvent in cytotoxic activity of *Acanthophora* against human cancer cells. Our current result would provide information regarding potential development of *Acanthophora spicifera* from Indonesian coastal areas in medicinal and pharmaceutical industries.

## MATERIALS AND METHODS

### Sample collection and extraction

Red macroalgae *Acanthophora spicifera* were collected from North West Lombok coastal area (8°24'11.7396"S, 116°41.9056"E), West Nusa Tenggara Province, Indonesia in the end of 2017. Macroalgae specimens were identified according to electronic algae database.<sup>10</sup> 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 5x volume of sample weight (w/v). Suspensions were then macerated by incubation in room temperature for 48h. Every 24h, 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. Same procedure was conducted to obtain methanol extracts.<sup>11</sup>

### Chemicals and reagents

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. The resulting pasty extracts were stored in a refrigerator at 4°C for further use. The extraction was centrifuged at  $15,000 \times g$  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  $15,000 \times g$  for 15 min, then obtained clear macroalgae extract sample.

### 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<sub>2</sub> 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 AS-MetOH were estimated by Lactate Dehydrogenase (LDH) assay and cell proliferation was measured by Trypan blue dye exclusion assay.<sup>12</sup> 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  $\mu\text{g/mL}$ ) of AS-MetOH and AS-EtOH. After 24 h incubation, a portion of the culture medium ( $\pm 100 \mu\text{L}$ ) was transferred to another 96-well plate, which

contained 2.5 mM NAD, 50 mM lithium lactate, 100 mM Tris (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 ( $A_{450 \text{ max}}$ ) was obtained from completely dead cells by addition of 1% Triton X-100 in the cell culture and minimum absorbance ( $A_{450 \text{ min}}$ ) was obtained from the control cell culture. Cytotoxicity was calculated by the following equation; Cytotoxicity (%) =  $(A_{450 \text{ treatment}} - A_{450 \text{ min}}) / (A_{450 \text{ max}} - A_{450 \text{ min}}) \times 100$ . 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 with maximum concentration of 200  $\mu\text{g/mL}$  for 72h. For early detection of apoptotic activity, cells were subjected to Hoechst 33342/PI viability staining.<sup>13</sup> Treated cells were stained 2  $\mu\text{L}$  volume of Hoechst33342 and incubated for 15 min in CO<sub>2</sub> 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  $\mu\text{g/mL}$  concentration of AS-EtOH and AS-EtOH for 3 days.<sup>14</sup> 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. DNA was analyzed by 1.5% agarose gel electrophoresis. Staurosporine (STS) was used as positive control.

### 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  $\pm$  SD. A *p*-value of less than 0.05 was considered to be statistically significant.

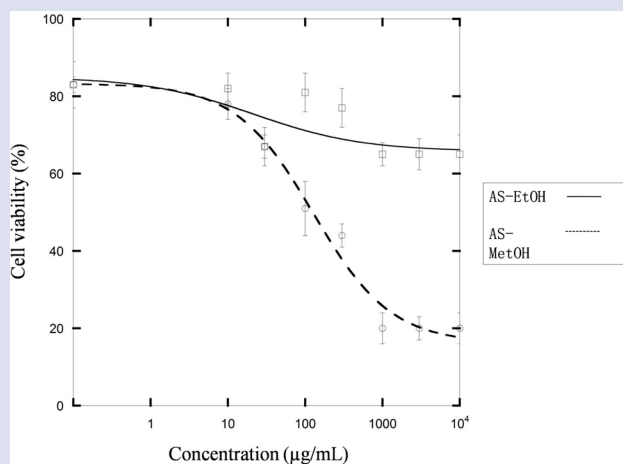
## RESULTS

### Cytotoxicity and antiproliferative assay

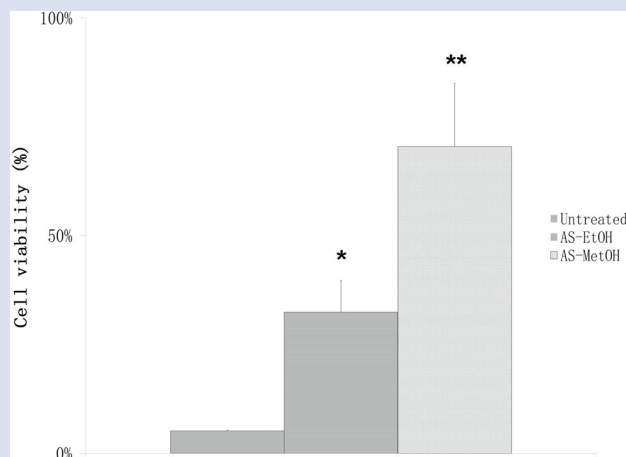
Cytotoxicity and antiproliferative effects of red seaweed AS-EtOH and AS-MetOH extracts were investigated via Lactate Dehydrogenase (LDH) assay against HeLa human cervical cancer cell line. Cytotoxic activity of *Acanthophora spicifera* methanol extracts increased in a dose-dependent manner (Sure 1). After 72h, the  $IC_{50}$  value of AS-MetOH crude extract was  $127.3 \pm 33.13 \mu\text{g/mL}$ . Whereas,  $IC_{50}$  value of AS-EtOH crude extract could not be determined in this study. AS-EtOH and AS-MetOH antiproliferative effects were determined with cell count by trypan blue exclusion assay. Figure 2 shows that untreated cells show high cell density with total cell count approximately  $80 \times 10^4$  cells/mL in 72h. Treated with AS-MetOH extracts resulted in suppressed cell density ( $23 \times 10^4$  cells/mL). In contrast, cell density in AS-EtOH treated cells ( $73 \times 10^4$  cells/mL) shows no antiproliferative activity.

### Hoechst3342-PI viability staining

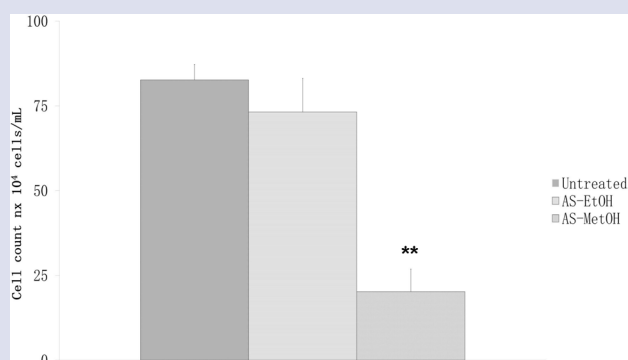
Cell morphology observation under fluorescence microscope shows that untreated HeLa cells shown normal elongated cell shapes (Figure 3). Viability staining with double staining Hoechst33342/PI stained live/dead cells; blue fluorescence dye Hoechst33342 and dead cells were stained red fluorescence dye Propidium Iodide (PI). These nucleic dyes would bind to specific regions in DNA, so stained particles are assumed



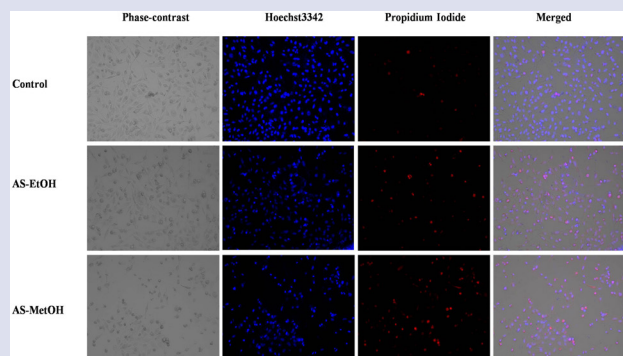
**Figure 1:** Cytotoxic assay of HeLa Incubated Cells AS-EtOH and AS-MetOH for 72h Analysed with LDH assay. Graph Fitting for Dose Response Analyses were Conducted with Kaleidagraph Software.



**Figure 4:** Dead Cell Percentage of HeLa Cells Treated with 200 µg/mL Concentrations of AS-EtOH and AS-MetOH for 72h. Dead Cell Percentages were Calculated with ImageJ Software. \*, *t*-test ( $p < 0.05$ ) Significantly Different; \*\*( $p < 0.001$ ) Highly Significant Compared to Control.



**Figure 2:** Antiproliferative Assay of HeLa Incubated Cells with AS-EtOH and AS-MetOH Extracts for 72h. 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.



**Figure 3:** Fluorescence microscopy observation of HeLa cells incubated with 200 µg/mL of AS-EtOH and AS-MetOH extract for 72h. Blue cells stained with Hoechst3342 indicate live/dead cells. Red cells stained with Propidium Iodide indicate dead cells.

to be cells. Untreated cells showed low percentage of cells emitting red fluorescence (Dead cells) (Figure 4). Treatment of HeLa cells with AS-EtOH appeared to induce significant percentage of dead cells compared to control. Furthermore, dead cell percentage was highly significant in HeLa cells treated with methanolic extract of *Acanthophora spicifera* compared to untreated cells. Overall, cells treated with AS-MetOH shown to experience reduction of cell size, cell shrinkage and the formation of blebs on cell surface resulted in the generation of apoptotic bodies.

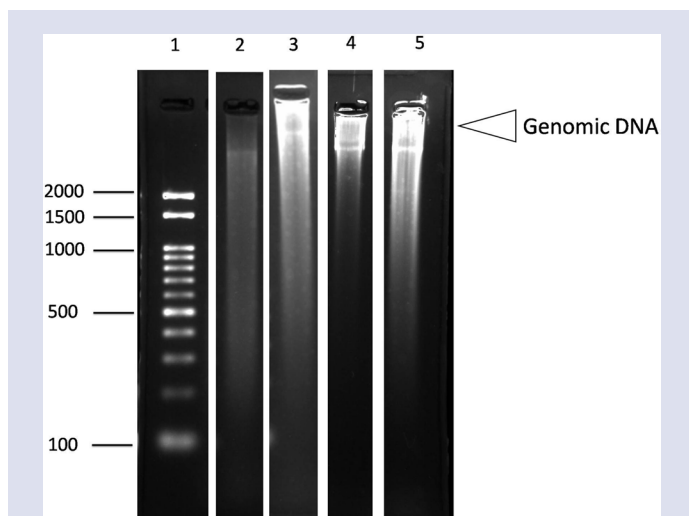
### DNA fragmentation

DNA fragmentation assay was used to determine the action of AS-EtOH and AS-MetOH extracts which induces cell death in HeLa cell *in vitro* models. As shown in previous results, AS-MetOH 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 AS-MetOH treated HeLa cells (Figure 5). In contrast, cells treated with AS-EtOH did not show indications of apoptotic activity.

### DISCUSSION

Several literatures have evidenced the potential implications of marine products such as macroalgae or seaweeds which exhibit antitumor activity.<sup>5,15</sup> Despite Indonesia is well known to exhibit high biodiversity of macroalgae, there remains limited information for their potential utilizations. Previous studies have reported potentials of *Acanthophora spicifera* ethanol extract anticancer potentials.<sup>3,9</sup> However, the cytotoxic activity presented is ineffective for its development as potential anticancer drug. Hence, in our study we conducted extraction of macroalgae *A. spicifera* with methanol solvent to evaluate its effects in cytotoxicity *A. spicifera* against HeLa cells.

The methanol extract of *A. spicifera* (AS-MetOH) shown significant anti-proliferative and cytotoxic activity and potentially induces apoptosis in HeLa cells with  $IC_{50}$  value of  $127.3 \pm 33.13$  µg/mL (Figure 1). In other hand, the ethanol extract of *A. spicifera* (AS-EtOH) cytotoxicity could not be determined with the applied concentrations in this study (0-200 µg/mL). Previous study has demonstrated low cytotoxic activity of *A. spicifera* crude ethanolic extracts in A549 human non-small lung carcinoma and HeLa cervical cancer cell lines with  $IC_{50}$  of  $666 \pm$  µg/mL and



**Figure 5:** DNA Ladder Assay for Detection of Apoptosis in HeLa Cells Treated with Macroalgae Extracts. HeLa Cells were Cultured in DMEM with 10% FBS and 200  $\mu\text{g}/\text{mL}$  Concentration of Extracts for 72h. Apoptotic Cells were Induced by 4  $\mu\text{M}$  Staurosporin (STS) as Positive Control. 5  $\mu\text{g}$  DNA of each Sample was Electrophorised on a 1.5% Agarose gel and the gel was stained by ethidium bromide. Lane 1: DNA ladder 1kb; Lane 2: AS-EtOH; Lane 3: AS-MetOH; Lane 4: Control (no macroalgae extract); Lane 5: STS (Positive control).

641  $\pm$  0.21  $\mu\text{g}/\text{mL}$  respectively. In general,  $\text{IC}_{50}$  values > 500  $\mu\text{g}/\text{mL}$  and at least 60% cell viability are considered as low cytotoxicity effect.<sup>16</sup> Our results revealed that AS-MetOH exhibit higher cytotoxic activity compared to AS-EtOH. Furthermore, antiproliferative assay with trypan blue cell count also revealed that cell proliferation is significantly inhibited by AS-MetOH treatment in HeLa cells for 72h (Figure 2).

Some studies on the effectiveness of extraction methods highlight that methanol extracts exhibit higher anticancer activity.<sup>17,18</sup> This result could be related to the presence of bioactive metabolites that are soluble in *A. spicifera* are possibly more effectively extracted in methanol solvent compared to ethanol. Treatment of *A. spicifera* methanolic extract was found significantly toxic in brine shrimps at 12 and 24 hr.<sup>19</sup> Furthermore, *in vivo* studies in cancer induced Swiss Albino mice administered with *A. spicifera* methanol extract (200mg/kg bw) significantly inhibited the cancer volume, packed cell volume, cancer cell count and haematological parameters in normal levels.<sup>20</sup>

The macroalgae *A. spicifera* crude extracts were further evaluated for cancer apoptotic death mode. Apoptosis is the common preliminary targeted mechanism for screening of anticancer potential from novel natural resources.<sup>21</sup> Moreover, apoptosis is a process which removes individual components of cells and avoids inflammatory effect which is normally associated with necrosis; thus potentially no toxicity to the normal surrounding cells.<sup>22</sup> To investigate whether the cytotoxic effects of the crude extracts were due to apoptosis, we treated the HeLa cells with AS-EtOH and AS-MetOH for 72h. After treatments, the cells were then subjected to double viability staining with Hoechst33342 and PI. Membrane permeable Hoechst33342 fluorescence stain stains the nuclei of both live and dead cell. However, Propidium Iodide is not permeable to the cell membrane.<sup>23</sup> Fluorescence stain PI only stains cells that has lost membrane permeability and binds to DNA by intercalating between the bases. Our results show that AS-EtOH and AS-MetOH induce morphological changes in treated HeLa cells. These changes such as irregular cell size and shape are more observable in AS-MetOH treated cells. Furthermore, cells which emit red fluorescence show higher

percentage in AS-MetOH (70%) treated cells compared to AS-EtOH treated cells (32%). Propidium Iodide (PI) stain could enter the cell during late-stage apoptosis apart that it also stains dead cells. Hence, cells emitting red fluorescence could possibly be assumed undergoing apoptosis.

Evaluation of apoptosis was further carried out by determining the DNA fragmentation as a result of apoptotic activity by caspase enzymes, indicative of the late stage of apoptosis.<sup>24</sup> HeLa cells treated with AS-MetOH showed characteristics of DNA fragmentation similar to positive control STS (Figure 5). However, DNA fragmentation activity was not observable in AS-EtOH treated cells. DNA fragmentation assay in HeLa cells treated with AS-EtOH and AS-MetOH were observed within maximum of 3 days, because after that all cells died.

## CONCLUSION

In conclusion, AS-MetOH exhibited significant cytotoxic and antiproliferative activity against HeLa cells compared to AS-EtOH. The cytotoxic effect of AS-MetOH appears to have induced apoptosis in HeLa cells. This study is the first to evaluate the potential apoptotic activity induced by macroalgae *Acanthophora spicifera*. Further investigation on *A. spicifera* methanol extract cytotoxic activity in a molecular and compound level would be reasonable for development as complementary and alternative anticancer agent.

## ACKNOWLEDGEMENT

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ABBREVIATIONS

**AS-EtOH:** *Acanthophora spicifera* Ethanol Extract; **AS-MetOH:** *Acanthophora spicifera* methanol Extract; **DMEM:** Dulbecco's Modified Eagle Medium; **DNA:** Deoxyribonucleic Acid; **EC<sub>50</sub>:** 50% Effective Concentration; **LDH:** Lactate Dehydrogenase; **NAD:** Nicotinamide Adenine Dinucleotide; **PI:** Propidium Iodide; **PMS:** Phenazine Methyl Sulfate; **STS:** Staurosporin; **WST-1:** Water Soluble Tetrazolium-1.

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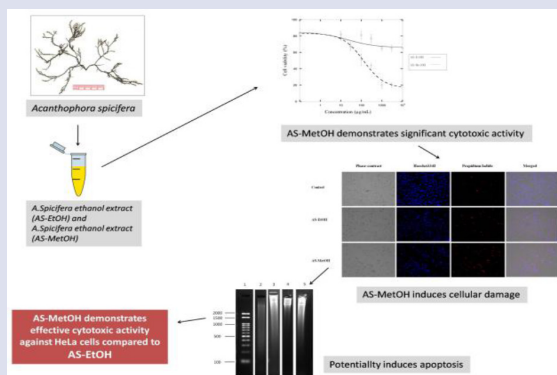
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## GRAPHICAL ABSTRACT



## SUMMARY

- Red Macroalgae *Acanthophora spicifera* (AS) has high abundance in Indonesian coastal waters. Several studies have evidenced potential anticancer activity of AS. However, previous study conducted with ethanol extract of AS resulted in low cytotoxic activity. In this study, we evaluated AS anticancer potential in a different solvent which is methanol. Current results show that AS Methanol Extract (AS-MetOH) demonstrates higher cytotoxicity compared to AS Ethanol Extract (AS-EtOH) in HeLa cells. Furthermore, treatment with AS-MetOH in HeLa cells was seen to induce apoptosis. In the other hand, AS-EtOH treatment apoptotic activity was not clear in our treatments. Further purification to obtain a potentially active and pure compound from *Acanthophora spicifera* crude methanol extract would be undertaken in the future for its potential development as natural anticancer agent.

## ABOUT AUTHORS



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