ABSTRACT

Objective: To investigate the in vitro cytotoxicity effect of the crude ethyl acetate extract of Cladosporium sp. on MCF-7, HeLa, and DU-145 cell lines.

Methods: In vitro cytotoxicity was evaluated by tetrazolium reduction assay. The percentage of cell inhibition was analyzed using probit analysis to obtain 50% inhibitory concentration (IC50). Morphological alteration of the cell lines after exposure with extract was observed under an inverted microscope.

Results: The ethyl acetate extract of the metabolite performed an anticancer activity for cancer cell line MCF-7, HeLa, and DU-145 with IC50 respectively 8.46 μg/ml; 9.87 μg/ml; and 98.03 μg/ml. The extract shows greater the anticancer activity and has strong antiproliferative on MCF-7 and HeLa cell line than DU-145. Confirmation morphological were observed under the inverted microscope showed a morphological change in cancer cells when incubated with the extract.

Conclusion: From the performed assay, the crude extract of Cladosporium sp. exhibit cytotoxic activity against MCF-7, HeLa, and DU-145.

Keywords: Cytotoxicity, Cladosporium sp., Endophytic, Fungus, MTT assay

INTRODUCTION

Nature has been an important source of novel anti-cancer drug leads, with increasing numbers of new compounds sourced from the marine environment. Macroalgae signify the second largest source of marine fungi after mangrove swamps [1]. Marine fungi have shown promising potential sources of new and proactive products as suggested by the chemical diversity of their secondary metabolites. As it seems that algae and their endophytic microorganism would be a good source of biologically active secondary metabolites [2] which have long been produced as an adaptation for specific functions in nature [3]. Some researchers have been able to isolate endophytic Cladosporium from different plant and macroalgae [4-11]. In this study, endophytes Cladosporium sp. was isolated from plant tissue host macroalgae Sargassum cinereum taken from the territorial waters of Pramuka Island, Indonesia.

The use of crude extracts of metabolite compounds produced by Cladosporium sp. has been shown to show anticancer activity [4]. Testing of several bioactive compounds successfully isolated from Cladosporium spp., such as taxol [4, 5], Sporiolides A and B [6], Cladosporolactam A [7], Anhydro-fusarubin, Methyl ether of fusarubin, Cladosporol I and C [8], Cladosporone A [9] can demonstrate the powerful antiproliferative effect of cancer cell lines, thus the potency of metabolite from Cladosporium sp. spp. can be developed as candidates of the anticancer drug.

In this study, using the human breast cancer adenocarcinoma cell line (MCF-7) that causes the most death in women has been studied for its sensitivity to metabolites from Cladosporium [4, 5, 11]. Type of other cancer cell lines cervical cancers (HeLa) that is commonly found in Indonesia. Human prostate cancer cells (DU-145) is the most common type of cancer in the elderly [12]. Both types of cancer cell lines have not reported any effects of proliferation with the use of extracts or active compounds from Cladosporium sp.

Considering the potency of the medicinal uses of Cladosporium sp., the objective of this study investigates the in vitro cytotoxicity of the crude ethyl acetate extract of metabolite produced by Cladosporium sp. on MCF-7, HeLa, and DU-145 cell lines.

MATERIALS AND METHODS

Fungal material

Cladosporium sp. EN-S01 was isolated from the marine brown algae Sargassum cinereum, collected from Pramuka Island, Kepulauan Seribu Marine National Park, Indonesia. This fungus was identified by morphological features, including the characteristic of ascospores and colonies The pure cultures were deposited in the Laboratorium of Microbiology, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran.

Cultivation and extraction

Cladosporium sp. was cultivated in 1 l Erlenmeyer flask containing saline water, Potato Dextrose Broth, CaCO3, yeast extract, and chloramphenicol. The culture was incubated at 25 °C for 5 d. The filtered broth was extracted with ethyl acetate then evaporated under vacuum in a rotary evaporator, to give 1 g of the dark brown viscous mass of the crude ethyl acetate extract.

Cell culture

Three human cancer cell lines were used to assess anticancer activity, MCF-7, HeLa, and DU-145 cell lines provided by Cell and Tissue Culture Laboratory, Teaching Hospital Universitas Padjadjaran. Human cancer cell lines were maintained as monolayer cultures in RPMI, supplemented with 10% Fetal Bovine Serum and 1% of the antibiotic solution under an atmosphere of 5% CO2 at 37 °C. Cell was trypsinized confluent. A stock solution of crude ethyl acetate extracts of Cladosporium sp. was prepared in 0, 1, 10, 100, and 1000 μg/ml. All the cells were incubated with the extract and dissolved in RPMI just before use, in order to maintain the same condition for all cell lines [13].

Tetrazolium reduction assay

The effect of anticancer from crude ethyl acetate extracts of Cladosporium sp., algae marine-derived fungus on MCF-7, HeLa, and
DU-145 cell lines was evaluated through micro-culture tetrazolium assay (MTT) and 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST). In this study, the crude extract was tested in vitro by using WST CCK-8 kit [14], while MTT kit was used for HeLa and DU-145 cell line. In principle, the use of WST reagent works similarly to MTT by reacting with mitochondrial succinic-tetrazolium reductase which forms formazan dyes [15].

The assay was done with plated the cells in 96-multiwell culture plates at a density of 0.8 x 10^4–1 x 10^4 cells/well. Twenty-four hours after plating, the medium was discarded and fresh medium containing the extract of Cladosporium sp. at different concentrations (1, 10, 100, and 1000 µg/ml) and control (0 µg/ml) was added. After 48 h incubation with extracts, the reagent of WST CCK-8 or MTT kit was added at a final concentration of 0.5 mg/ml and incubated for 2 h, furthermore, the medium was removed.

To evaluate the effect on cell proliferation, the absorbance at the beginning of incubation was subtracted from all the experimental condition used, including the control. The results are expressed as the percentage of cell proliferation relative to control (untreated cells) carried out in duplicate. Percentage of cell viability (CV) was calculated manually using the formula:

\[
\% \text{ Cell Inhibition} = 100 - \frac{\text{Abs (sample)}}{\text{Abs (control)}} \times 100
\]

Positive values (between 0 and 100%) can be interpreted as inhibition of cell proliferation. A dose-response curve were plotted to enable the calculation of IC_{50}. The IC_{50} values were determined using Probit Analysis. IC_{50} corresponds to the concentration of the extract that decreases the number of viable cells by 50%. In this case, the absorbance in the control corresponds to 100% viability.

Morphological analysis

Morphological observation of cell treated with crude ethyl acetate of fermented Cladosporium sp. extract from cytotoxicity study was done to determine the changes induced by the extracts. Morphological alteration such as cell shrinkage, membrane blabbing, rounded and detached cells were observed for confirmation effect of cell death.

RESULTS AND DISCUSSION

Antiproliferative effects of MCF-7, HeLa, and DU-145 cell lines

In vitro cytotoxic activity of Cladosporium sp. ethyl acetate extracts in MCF-7, HeLa, and DU-145 cell lines were cultured in the absence and presence of the crude extract of multiple concentrations. As shown in (table 1).

| Concentration (µg/ml) | % Cell inhibition |
|-----------------------|------------------|
|                       | MCF-7            | HeLa             | DU-145                |
| 0                     | 0.00             | 0.00             | 0.00                  |
| 1                     | 31.49±9.90       | 32.66±5.44       | 6.31±10.18            |
| 10                    | 51.42±5.25       | 50.10±4.39       | 28.32±21.82           |
| 100                   | 55.18±0.73       | 54.34±1.42       | 50.24±6.88            |
| 1000                  | 53.07±4.89       | 57.99±3.48       | 71.14±5.66            |

Result represent mean±standard deviation (n=3)

It was observed, the IC_{50} (concentration of the extract that decreases the number of viable cells by 50%) for each cell line was calculated from the dose-response curves (fig. 1-3). The crude extract of ethyl acetate exhibited significant activity against the MCF-7 and HeLa cell line with an IC_{50} values of 8.4 µg/ml and 9.87 µg/ml, however, DU-145 showed the only IC_{50} value of 98.03 µg/ml. Results showed that the fungal crude extract could significantly inhibit the viability of the cancer cells and have potential anticancer activity.

![Fig. 1: Effect on antiproliferative of Cladosporium sp. on MCF-7 cell line](image1)

![Fig. 2: Effect on antiproliferative of Cladosporium sp. on HeLa cell line](image2)
Fig. 3: Effect on antiproliferative of Cladosporium sp. on DU-145 cell line

The reduction in the number of cells was found to suppress the cell proliferation and indicated with having damaged its cell structure. Confirmation by observing the morphological changes of normal cells and their effects after exposure with extracts containing anticancer.

The living MCF-7 and HeLa, and DU-145 cell lines normally observed in epithelial and polygonal shape. Treatment of the cell lines with the ethyl acetate extract of Cladosporium sp. differ the appearance of the normal cells found to be irregular, aggregate, spherical in shape indicating damaged cells and spreading patterns were constrained. The in vitro anticancer activity of the extract in MCF-7, HeLa, and DU-145 cells was mainly due to the induction of cell death. The characteristic is common to several chemotherapeutic drugs, which reveal an anticancer activity mainly due to their ability to induce DNA damage; if such DNA damage is not properly repaired, its accumulation ultimately ensues in cell death [16].

Fig. 4: (a) normal cell lines and (b) morphological alteration at 100 μg/ml (Magnification 100x)

DISCUSSION

The use of anticancer extracts from Cladosporium spp. have been evaluated in some of the most deadly cancer cell lines, such as colon cell lines (HCT-116 and HCT-15), breast (MCF-7 and T47D), prostate (PC-3), cervix (HeLa), Prostate (DU-145), Mouse lymphocytic leukemia (L1210) and Leukemia (K-562), Lung (A-549 and H446), Hepar (Huh7), Liver (LM3). Table 2 showed cytotoxicity of the compounds and crude extract of metabolite produced by Cladosporium sp.

This study to investigate the cytotoxicity of the crude extract of metabolite produced by Cladosporium sp. in vitro MCF-7, HeLa, and DU-145 cell lines. Similar studies using plant were reported. IC50 of Monarda citriodora on colon (HCT-116), breast MCF-7, prostate (PC-3) cell lines were<10 μg/ml. The present study contributes to evidence that the metabolites produced by endopytic fungus Cladosporium sp. isolated from marine macroalgae have potential anticancer activity on in-vitro MCF-7, HeLa, and DU-145 cell lines. Furthermore, this study is the first that evaluates antiproliferative effects on cell lines cervical cancers (HeLa) from a crude extract of Cladosporium sp.
Cytotoxicity of crude extracts from *Cladosporium* sp which isolated from the terrestrial plant [17] proved anti-cancer activity with IC_{50} value was≤10 μg/ml. A similar result was obtained in this study, the use of crude extracts from *Cladosporium* sp. isolated from marine macroalgae *Sargassum cinerereum* showed toxicity, lower than 10 μg/ml except for prostate cancer line DU-145 IC_{50}. For the best of our knowledge, the application of crude extracts or purified-compounds against anticancer prostate line DU-145 cells has not been previously reported. The effectiveness of anticancer to DU-145 cell line is very important to be studied further and needs to be tested by using isolated compounds.

Data from our study can be more convincing for using the ethyl extract to produce the anticancer active compound from *Cladosporium* spp. The results obtained hence confirm that the fungus has significant anticancer potential as stated in the other reports about the *Cladosporium* [4-11]. Purification and isolation of anticancer compound from *Cladosporium* spp. have been studied, some were reported that they are able to increase the effectiveness for inhibiting the growth of cancer cell. Some endophytic *Cladosporium* genus from plants are known to produce several compounds with the anticancer activity, such as cladosporone [10] and taxol [11]. Shigemori et al. (2004) have reported that isolation of macroalides from endophytic *Cladosporium* sp. derived brown alga *Actinotrichia fragilis*, namely, sporiolides A and B. The results showed that sporiolides A and B showed an IC_{50}, respectively, 0.13 μg/ml and 0.81 μg/ml in L1210 (mouse lymphocytic leukemia) [6].

Cao et al. (2015) have reported the isolation of bicyclic lactam, namely *Cladosporiolactam A* exhibited promising cytotoxic activity against HeLa (cervical cancer cell line) with IC_{50} value of 0.76 μg/ml [7]. Khan et al. (2016) have reported the isolation of naphthoquinones from endophytic *Cladosporium* sp. derived *Rauwolfia serpentina*, namely, anhydrofusarubin and methyl ether of fusarubin. The results showed that anhydrofusarubin and methyl ether of fusarubin showed an IC_{50}, respectively, 3.97 μg/ml and 3.58 μg/ml against K-562 (human leukemia cells) [8]. Li et al. (2017) reported cytotoxic activity from marine algal-derived endophytic fungus *Cladosporium cladosporioides* EN-399 compounds. *Cladosporol* I showed cytotoxicity against A549 (human lung adenocarcinoma), Huh7 (human hepatocarcinoma), and LM3 (human liver cancer) with IC_{50} values of 5.0, 1.0, and 4.1 μg/ml, respectively, and *Cladosporium* C showed activity against H446 (human lung cancer) with IC_{50} value of 4.0 μg/ml [9].

have been struggling for new methods in which to improve anticancer compound production to meet the drug demand.

**CONCLUSION**

In summary, the crude extract of endophytic fungus *Cladosporium* sp. from marine macroalgae *Sargassum cinerereum* demonstrates a high potential anticancer activity in breast adenocarcinoma (MCF-7), human epithelial carcinoma (HeLa), and human prostate carcinoma (DU-145). Further, investigations will be interesting to find medicinal compound and should focus on isolating the potential molecule which responsible for the activity of anticancer.

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**AUTHORS CONTRIBUTIONS**

Conceived and designed the experiments: APW, DH, PA, Performed the experiments: RRINE, Analyzed the data: APW, RRINE, M, Wrote the paper: APW, RRINE

**CONFLICTS OF INTERESTS**

All authors have none to declare

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**Table 2: Cytotoxicity of the compounds and crude extract of metabolite produced by Cladosporium spp**

| Species Endophytic source | Solvent     | Compound   | Cell line          | IC_{50} Ref. |
|---------------------------|-------------|------------|--------------------|--------------|
| *C. tenuissimum* Plant *Monarda citriodora* | Methylen chloride | Crude extract | Golen (HCT-116) | <10 μg/ml [4] |
| *Cladosporium* sp. Marine macroalgae *Sargassum cinerereum* | Ethyl acetate | Crude extract | Breast (MCF-7) | <10 μg/ml [4] |
| *C. oxysporum* Plant *Moringa oleifera* | Dichloromethane | Taxol | Golvex (HeLa) | 8.46 μg/ml This study |
| *Cladosporium* sp. Marine macroalgae *Actinotrichia fragilis* | Ethyl acetate | Sporiolides A | Breast (DU-145) | 1.0 μg/ml |
| *Cladosporium* sp. Coral, *Anthogorgia ochracea* | Ethyl acetate | Cladosporilactam A | Breast (T47D) | 2.5 μM |
| *Cladosporium* sp. Plant *Rauwolfia serpentina* | Ethyl acetate | Anhydrofusarubin | Leukemia (K-562) | 3.97 μg/ml [8] |
| *C. cladosporioides* Marine macroalgae *Laurencia okamurai* | Ethyl acetate | Methyl ether of fusarubin | Leukemia (K-562) | 3.58 μg/ml |
| *Cladosporium* sp. Mangrove *Kandelia candel* | Ethyl acetate | Cladosporol C | Lung (A-549) | 5.0 μg/ml [9] |
| *C. oxysporum* Plant *R. serpentina* | Ethyl acetate | - | Hepar (Huh7) | 4.1 μg/ml [10] |
| | | Taxol | Liver (LM3) | 4.0 μg/ml |
| | | Crude extract | Lung (H446) | 4.0 μg/ml |
| | | - | - | - |
| | | - | - | - |
| | | - | - | - |
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