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Research Article

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Anticancer potential of Seagrass leaves *Cymodecea serrulata* CRUDE extract on HeLa cell

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ABSTRACT

Seagrass is a plant that lives in tidal areas, possesses unique secondary metabolites, which have roles as anticancer bioactive compounds. The aim of this research was to determine the potential of seagrassCymodecea serrulatafresh leaves extract as an anticancer agent. The method used was experimental method through continuous extraction towards seagrass leaves using n-hexane (non-polar), ethyl acetate (semi polar) and ethanol (polar). Extract obtained was analyzed for its anticancer activity by in vitro using HeLa cell. Results showed that non-polar extract has no anticancer activity, while semipolar and polar extract showed their potential as an anticancer agent. HeLa cell lethality level by semi polar extract was higher than polar extract, but not significantly different with cancer medicine doxorubicin. HeLa cell lethality by semi polar extract, polar extract and doxorubicin are 48.11%, 15.32 % and 48.75%, respectively. Ethyl acetic crude extract of fresh seagrass contain phytochemical compounds of alkaloid, terpenoid, polyphenol and flavonoid.

Keywords: crude extract, seagrass*Cymodecea serrulata*, anticancer, lethality, phytochemical

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INTRODUCTION

Cancer disease has been a major health problem in the world and also in Indonesia. Based on WHO data on 2013, cancer incidence increased from 12.7 million cases (2008) to 14.1 million cases (2012). It is predicted that in 2030, cancer incidence could reach up to 26 millions people, in which 17 millions people could die because of cancer, particularly in under-developed countries and developing countries. In Indonesia, prevalence of cancer disease is also quite high. Based on data from *Riset Kesehatan Dasar (Riskesdas)* in 2013, the prevalence of tumor/cancer in Indonesia was 1.4 per 1000 persons, or about 330,000 persons [1].

Several treatments to prevent and cure cancer have been done, such as radiotherapy, chemotherapy, or using synthetic medicine. In general, anticancer therapy has been felt to be sufficient to give good results, but it has side effects and the costs are quite [2]. Radiotherapy and chemotherapy have limitations. The effectiveness of rays used for radiotherapy will decrease along with the increase of tumor size, meanwhile the increase of dosage given to the level exceeds its toxicity level will give effect to normal human tissues and organs. The use of chemical drugs such as chemotherapy kill not only the tumor cells but also damage blood cells, which causes the decrease in immunity or even death, resulting from complications due to side effects of drugs [3]. Several publications data also stated that cervical cancer cells have become resistant towards treatment using radiation and chemotherapy[4] [5].

Therefore, many people have turned to traditional medicines that are considered more safe and economical, because it uses natural ingredients. Natural ingredients contain several active compounds which give pharmacological effects. In general, those active compounds are secondary metabolites [6][7]. Secondary metabolites have been known as sources of medical therapy, for example as antibacterial and anticancer medicines, etc[8][9].

Majority of secondary metabolites are synthesized by organisms to adapt to their environment, thus, the search of secondary metabolites that are able to act as anticancer bioactive mainly focused on organisms that live in extreme environmental condition.

One of extreme environments is at tidal area. Organisms which live at tidal area and have been reported to contain anticancer bioactive compounds are sponges and their endophytic microbes [10], Ascidia (*sea squirt*)[11][12], microbes that associated with sea biota, such as marine derived fungi[13][14][15], mangroves and their endophytic microbes [16][17][18][19], and seagrass and its endophytic microbes [20][21].Symbiotic or ephytic bacteria on seagrass, a term for bacterial colony that lives, grows and associates with seagrass, has been reported to contain similar bioactive compounds to its symbiont[20][21].

Seagrass is a hydrophyte that lives in tropical or subtropical tidal coastal areas. Around the world, there are 52 types of seagrass, 15 of which presents in Indonesia. Several researches showed that seagrass is potential as antioxidant, anticancer and antibacterial agents. Compounds in seagrass that are suspected to be able to inhibit cancer cell proliferation and inactivate pathogenic bacteria are flavonoid, saponin, steroid, terpenes, tannin and alkaloid. [22] stated that seagrass*Enhalus acoroides* contains stigmasterol, sitosterol and alkaloid. Seagrass*Thalassia testudinium* contains glycosides and phenol compounds which are potential to act as antifungi and anticancer, while seagrass*Halodule uninervis* contains steroid compounds which acts as antibacterial and anticancer.

[23] stated that crude extract from seagrass*Thalassia emprichii*, *Cymodocea serrulata* and*Enhalus acoroides*had high phenolic content. Moreover,[24] reported the cytotoxicity of crude extract from seagrass*Enhalus acoroides*and*Cymodocea serrulata*toward*Artemia salina*at concentration of 404.88 ppm and 136.398 ppm, respectively. [25] stated that the highest phenolic content is on the leaves part.

One of seagrass that can be found in tidal coastal area in Indonesia is *Cymodecea serrulata*. Since other types of seagrass were reported to contain anticancer bioactive compounds, another research to determine the potential of seagrass*Cymodecea serrulata*as a source of anticancer bioactive compounds should also be conducted.

EXPERIMENTAL SECTION

Materials and equipment

The materials used were fresh seagrass *Cymodecea serrulata*leaves which were obtained from coastal area Sanur – Bali – Indonesia, ethanol, ethyl acetate, n-hexane (Merck-Germany), aquadest, filter paper Whatman No.1, trypan blue, RPMI 1640 (Roswell Park Memorial Institute), fetal bovine serum(FBS), dimethyl sulfoxide (DMSO), Penicillin-streptomycin, $NaHCO_3$, D-PBS, trypsin-EDTA, MTT reagent, and detergent reagent.

Equipment used in this experiment were rotary evaporator (Buchii R-124), grinder (Philips HR2108), aluminium foil, measuring cylinder, funnel , flask, erlenmeyer (Iwaki Pyrex), analytical balance (Nettler ToledoAB204-S), laminar air flow (Esco), hot plate (Thermoline), vortex mixer (Thermoline), cryo tube, centrifuge tubes, centrifuge (Hermle Z206A), micropipette and tip (Thermo Scientific), filter 0.2 µm, incubator with 5% CO_2 humidity at 37^oC (Memmert), microscope (Olympus CX-3), haemocytometer (Brand), microplate plate reader 650 and 750 nm, serological pipette (Brand), and Spectrophotometer (Thermo Scientific Genesys 20).

Research Methods

The method used in this research was experimental method by extracting bioactive compounds from fresh seagrass and assessing anticancer activity from obtained extract. Extraction process (A) was performed continuously using non-polar solvent n-hexane (A1), semi polar solvent ethyl acetate (A2) and polar solvent ethanol (A3). Each obtained extract was assessed for its anticancer activity on HeLa cell, using dosage of 100 µg/mL (B1), 75 µg/mL (B2), and 50 µg/mL(B3). Based on these treatments, the first stage of this research was designed using Randomized Full Factorial Design with three replications for each treatment.

Seagrass Leaves Extract Preparation [26]

Leaves from fresh seagrass samples, which were obtained from SanurBeach, Bali - Indonesia, were washed using fresh water, drained, cut and size-reduced using blender. Then, leaves were added with hexanee solvent with ratio of 1:5 (b/v) and macerated for 3x24 hours at room temperature (30^oC). The maceration results were filtered using Whatman no.1 filter paper to obtain filtrate and residue. The filtrate was evaporated using rotary evaporator to obtain hexane extract (non polar extract). The residue was then added with ethyl acetate with ratio of 1:5 (b/v) and macerated for 3x24 hours at room temperature. The maceration results were filtered using Whatman no.1 filter paper to obtain filtrate and residue. The filtrate was evaporated using rotary evaporator to obtain ethyl acetic extract (semi polar extract). The residue was then added with ethanol with ratio of 1:5 (b/v) and macerated for 3x24 hours at room

temperature. The maceration results were filtered using Whatman no.1 filter paper to obtain filtrate and residue. The residue was discarded and the filtrate was evaporated using rotary evaporator to obtain ethanolic extract (polar extract). Non polar, semi polar and polar extract were treated with Nitrogen and stored in freezer for analysis.

Cytotoxicity Assessment on Extract towards HeLa Cell Using MTT Assay [27]

The amount of 100µL HeLa cell was added into wells using media control, cell control, positive control of doxorubicin, and seagrass leaves extracts (ethyl acetic, n-hexane and ethanolic extracts). Wells were incubated for 24 hours at 37^{o}C, with CO_2 supply of 5 ml/minute. After 24 hours, 10 µL of MTT (3-(4,5-dimethyltiazoly-2)-2,5 diphenil tetrazolium bromide) reactant was added. Then, it was incubated again for 4 hours inside CO_2 incubator, after which the reaction of MTT was stopped by adding 100µL of 10% Sodium Dodecyl Sulfate (SDS). The incubation continued for 12 hours at room temperature; then absorbance of each well was measured using spectrophotometer at wavelength of 570 nm.

Microplate that has been incubated for 12 hours at room temperature was assessed using MTT assay, and then HeLa cell lethality was calculated by measuring absorbance of each well using ELISA microplate reader spectrophotometer with wavelength of 570 nm. Result of HeLa cell death was observed by color change from yellow to formazan blue crystals. The lethality of cells was calculated using the following formula:

$$\text{Cell lethality (\%)} = \frac{\text{(Cell absorbance –Medium absorbance) – (Treatment absorbance-Medium absorbance)}}{\text{(Cell absorbance –Medium absorbance)}} \times 100\%$$

RESULTS AND DISCUSSION

Yield of Seagrass Leaves Crude Extract

Table 1.Yield of fresh Seagrass(*C. serrulata*) leaves crude extract

Crude extract	Yield (%)
N-hexane	2.2 ±1.1[a]
Ethyl acetic	45.9±2.0[b]
Ethanolic	75.7±3.2[c]

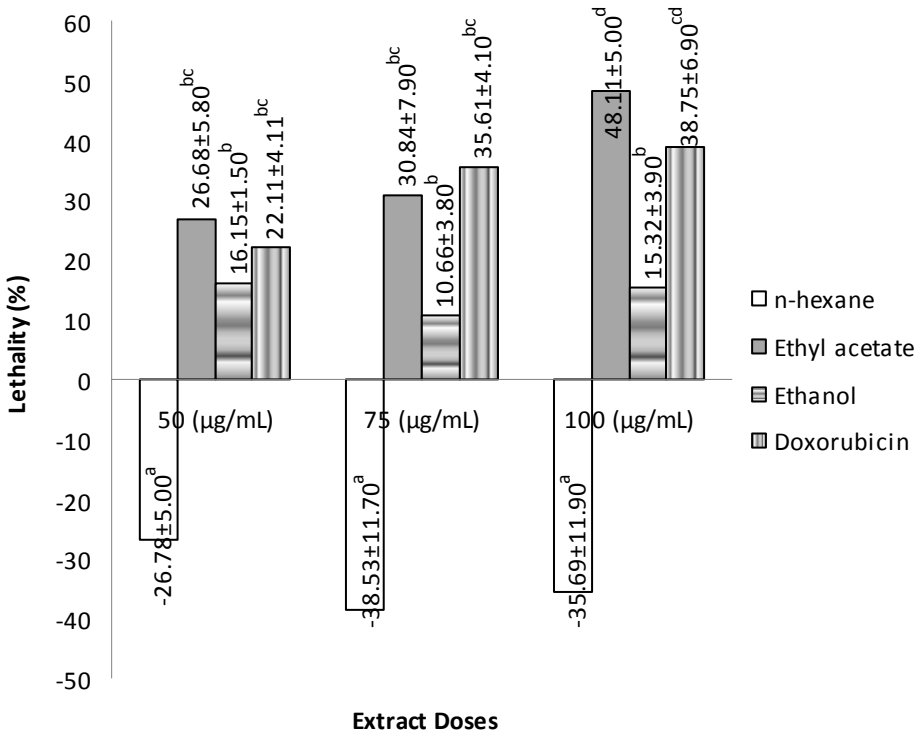
Notes: Different superscript letter notation shows significant difference (p<0.05)

Table 1 shows that the highest yield is obtained from ethanol extract, followed by ethyl acetic extract, and the lowest is n-hexane extract. The amount of yield correlated with solvent polarity properties and sample condition. Fresh seagrass leaves contain quite high moisture content; therefore the compounds mostly are polar. Since ethanol is polar, relatively highly soluble and has low boiling point [28], it could produce the highest yield extract. The lowest yield was obtained from n-hexane; it is influenced by moisture content of sample, solvent polarity properties, and the amount of non polar compound in sample. The high amount of water, which is polar [29], could inhibit the diffusion of n-hexane into the sample. As a result, non polar compounds, such as fatty acids, essential oils and terpenoid, could not be fully extracted. According to [30], bioactive compounds that are extracted from an ingredient depends on the polarity of solvent used. Compounds that are bound to polar solvent are alkaloid, amino acids, polyhydroxysteroid, and saponin; compounds that are bound to semi polar solvent include peptide and dipeptide; and compounds that are bound to non polar solvent are hydrocarbons, fatty acids and terpenes. Moreover, [31] stated that high surface area could increase the contact between solvent and particles of an ingredient. However, since the size of samples is similar, this factor did not influence the yield.

Citotoxicity of Fresh Seagrass Leaves Crude Extract Towards HeLa Cell

Cytotoxicity was measured using MTT assays and expressed in percentage of lethality. Negative lethality percentage shows no cell death, while positive value shows HeLa cancer cell death.

Analysis of variance results show that types of solvent used in extraction, dosage of extract and the interaction between both factors significantly affected the lethality of HeLa cell ($p<0.05$). The brief result from post hoc test using *Tukey* can be observed at Figure 1.



Notes:
- Different superscript letter notation shows significant difference ($p<0.05$)
- Positive control :doxorubicin 100 µg/ml; 75 µg/ml;50 µg/ml.

Figure 1. HeLa cell lethality by fresh seagrass leaves crude extract

Figure 1 shows that non polar extract (n-hexane extract) from seagrass leaves has no anticancer properties, while semi polar extract (ethyl acetate extract) and polar extract (ethanol extract) have anticancer properties. Anticancer properties, shown by percentage of lethality, were higher on ethyl acetic extract compared to ethanolic extract. This indicates that anticancer compounds in seagrass leaves are mostly semi polar. Lethality properties from ethyl acetic extract were similar to doxorubicin. Consequently, ethyl acetic extract from fresh seagrass leaves are the most potential to become anticancer medicine.

From another research, solvent that could extract anticancer compounds were n-hexane[32]. Anticancer compound that is non-polar is antioxidant [33]. In fresh seagrass leaves, n-hexane extract has no anticancer activity. This might be because n-hexane is non-polar, while fresh seagrass leaves still contain high amount of water that could inhibit n-hexane to difuse into sample [34]. Therefore, polar active compounds could not be extracted.

Phytochemical compounds that are present in ethyl acetic extract of fresh seagrass leaves are alkaloid, terpenoid, polyphenol and flavonoid (Table 2). Flavonoid compounds are suspected to possess anticancer activity. [35] suggested that flavonoid compounds that come from phenolic groups, such as morin, fisetin, quercetin, myricetin, and taxifolin, could destroy the growth of cancer cell at low concentration and stimulate apoptosis at high concentration. Therefore, further refining is required to determine anticancer compounds in fresh seagrass leaves.

Table 2.Screening of phytochemical compounds in fresh seagrass leaves extract

Compound	Activity
Alkaloid	+
Terpenoid	+
Steroid	-
Tannin	-
Polyphenol	+
Flavonoid	+

Table 2 shows that seagrass leaves crude extract contains flavonoid, polyphenol, alkaloid and terpenoid. These compounds are secondary metabolites from seagrass leaves that can be used as anticancer, antibacterial, anti-inflammation and antifungal. Secondary metabolites are produced by plants as a self defense mechanism from

predator and bad environmental conditions. Some compounds which are mostly used as anticancer are flavonoids, terpenoids, and phenols.

Flavonoid compounds are able to inhibit the process. Inhibition occurs at initiation, promotion or progression stage through molecular mechanisms, such as inactivation of carcinogenic compounds, anti-proliferative, inhibition of angiogenesis and cell cycle, apoptosis induction and antioxidant activity [36]. Mechanism of anticancer activity from polyphenol is by defending cells from DNA damage by preventing free radicals, decreasing tumor cell proliferation, inducing apoptosis, and inducing protein in signal transduction pathway, such as protein activator 1, phosphatidyl inositol 3-kinase, p7026-K, and mitogen-activated protein kinase[37].

Terpenoids also play important roles in reducing cancer cell proliferation and inducing apoptosis. Terpenoids can inhibit G2 cell cycle to prevent mitosis. In mammals, there are two types of topoisomerase enzymes, i.e. type I which cuts single strand of DNA and type II which cut double strands of DNA. In this case, terpenoids as topoisomerase enzyme inhibitor will damage DNA and induce apoptosis[38].

Phenolic also has anticancer activity. Phenols can soluble in polar and semi polar solvents. Phenolic compounds can destroy HeLa cell growth and breast cancer cell T47D. Caffeic acid in phenols can decrease expression of anti-apoptosis protein, to induce apoptosis [39].

Phenol and alkaloid are the most commonly found compounds in nature and come from plants. Alkaloid from red fruit, God's crown and *Temu putih*(*Curcuma zedoaria*) contain alkaline nitrogen atom and has physiological activity as anticancer agent [10]. Phenols from Kedadai leaves(*Ficus variegata*,Blume) was also reported to possess anticancer properties [40].

CONCLUSION

1. The suitable solvent to extract anticancer bioactive compounds from fresh seagrass is semi polar solvent, ethyl acetate. Ethyl acetic extract contains phytochemical, i.e. alkaloid, terpenoids, polyphenol and flavonoids.
2. Ethyl acetic extract of100µg/mL gives higher lethality level towards HeLa cell compared to ehtanolic extract. It also gives similar lethality level compared to cancer medicine, i.e. doxorubicin. Lethality level of ethyl acetic extract, ethanolic extract and doxorubicin are 48.11%, 15.32 %, and 4875%, respectively.

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