ABSTRACT

Introduction: In this century, cancer has increased in incidence and become one of the deadliest diseases in the world. However, to date, lung cancer treatments are still not fully effective, quite expensive and very exhausting for the patient. Eucheuma cottonii is an abundant marine red macroalgae in Indonesia which have a potential anti-lung cancer properties. Aim of this research is to determine phytochemical profile of Eucheuma cottonii extracts, as well as to evaluate its antioxidant and cytotoxic effects on Lung A-549 cancer cells. Methods: Eucheuma cottonii extracts were identified for its phytochemical profiles, antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, and cytotoxic activity on lung A-549 cells by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Results: Phytochemical analysis revealed that Eucheuma cottonii contains metabolites of triterpenoid and alkaloid. Antioxidant activity evaluation showed ethanol extract of Eucheuma cottonii has IC_{50} value of 559.76 μg/mL against DPPH free radical. Whereas cytotoxicity evaluation showed that ethanol extract and ethylacetate extract of Eucheuma cottonii have cytotoxic effects on Lung A-549 cancer cells, with IC_{50} value of 251.73 μg/mL and 261.41 μg/mL, respectively. Conclusion: These results suggesting that Eucheuma cottonii extract could be further developed as a natural anti-lung cancer agent. Key words: Eucheuma cottonii, Phytochemical, Antioxidant, Cell line study, Lung A-549 cells.

INTRODUCTION

Cancer is abnormal cell growth and has become one of the major non-communicable diseases (NCDs) that has reduced people life expectancy in this century.1,2 In 2018, cancer has increased by 18.1 million cases and has caused 9.6 million the total death of the world’s population so that it becomes second deadliest disease in the world. One of the most common and deadly cancers for both men and women in the world is lung cancer, with an incidence rate of 11.6% of total cancer cases and contributes 18.4% of total cancer deaths.3 In Indonesia, lung cancer is the highest cancer incidence in men and the first leading cause of death from cancer in men (21.8%) and second in women (9.1%)4, which prompted us to conduct specific cell line study of marine seaweed on lung cancer. This high incidence of lung cancer in Indonesia reached a prevalence of 36.3% in 2013, that is caused by high smoking behavior. It is known that smoking is the biggest risk factor (80-90%) of lung cancer. Thus, Indonesia places the third rank in the world for number of smokers.5,6

Treatments of lung cancer are surgery, chemotherapy, radiation and targeted therapy.7 However, these various treatments have some side effects, the emergence of resistance, toxicity, and the prognosis of treatment is still low with an average 5-year survival rate around 16%.7 Based on these facts, discovering of more effective, less side effect and inexpensive anti-lung cancer agent is needed. Seaweed or marine macroalgae are the marine species which have been commonly used as food and traditional medicine, especially in the Asian continent.4 Marine algal had shown biological activities as anti-inflammatory, anticoagulant, antioxidant, antiviral and anticancer, so that it has become promising candidate for the new bioactive compound used in medicines.8,9 One species of the marine red macroalgae which potential to be developed as an anticancer agent is Eucheuma cottonii. Some previous researchers has reported that Eucheuma cottonii demonstrated anticancer activity against MCF-7 breast cancer cells, HCT-116 colorectal cancer cells, and HeLa cervical cancer cells.10-13 Beside that, Eucheuma cottonii are also widely cultivated in Indonesia, including in West Papua, with production level reached 6549 tons per year.14 The anticancer activity and the large production of Eucheuma cottonii in Indonesia has encouraged us to carry out phytochemical analysis, antioxidant activity, and cell line study of nutritious Eucheuma cottonii against lung A-549 cancer cells.

test procedure was carried out according to the method provided. The phytochemical Phytochemical test are used to determine thesecondary metabolites. Maceration and extraction technique of Eucheumacottonii is adopted. Amount of 100 g of Eucheumacottonii dried powder was then macerated with 500 mL of n-hexane in a closed tube for 24 hours by occasional shaking. This maceration is done three times, the mixture was filtered. The filtrate is evaporated to give n-hexane extract of Eucheumacottonii. Whereas the solid were macerated three times with 500 mL ethyl acetate for 24 hours. The mixture was filtered, the filtrate is collected and evaporated to produceethylacetate extract of Eucheumacottonii. The solids were then macerated three times with 500 mL ethanol for 24 hours. The mixture was filtered, the filtrate is collected and evaporated to generate ethanol extract of Eucheumacottonii. Phytochemical test are used to determine the secondary metabolites containing in the extracts of Eucheumacottonii. The phytochemical test procedure was carried out according to the method provided by Harborne (1987). Phytochemical denotes all the following mentioned parameters. Test for saponin is carried out by shaking vertically 10 ml of the sample solution in a test tube for 10 seconds and left it still for 10 seconds. Formation of stable foam as high as 1-10 cm for 10 minutes or more indicates the presence of saponin metabolites. Addition of 1 drop of HCl 2N is done to ensure the foam still does not disappear, which confirm the presence of saponin. Test for flavonoid is carried out by evaporating 1 ml of the sample solution and continuing by adding of acetone, boric acid powder and oxalic acid. After that, the mixture is put into warm water at 60°C for 15 minutes. The mixture was then added with 10 ml of ether and observe it in UV light at 366 nm. The appearance of the yellow fluorescence in the UV light indicates the presence of flavonoid.

Test for tannin is carried out by reacting 1 mL of sample solution with 10% of FeCl₃ solution. Formation of dark blue or dark green color indicates the presence of tannin.

Test for glycoside is carried out by evaporating 1 mL of solution in warm water at 60°C for 15 minutes, then dissolving it into 5 mL of anhydrous acetic acid. Subsequently, concentrated sulfuric acid (10 drops) was added into the mixture. Formation of blue or green deposits indicate the presence of glycosides.

Test for triterpenoid and steroid were carried out using the Liebermann-Burchard reaction. Sample solution (2 mL) was evaporated in a porcelain glass. The residue was dissolved with anhydrous chloroform (0.5 mL) and acetic acid (0.5 mL). After that, 2 mL of concentrated sulfuric acid is added into the mixture through the tube wall. The formation of brown or purple rings indicates the presence of triterpenoids. Meanwhile, the formation of a blue green ring indicates the presence of steroids.

Test for alkaloid is done by evaporating 2 mL of sample solution on a porcelain. The residue were then added 2N of HCl (5 mL) and the mixture is divided into 3 different tubes. The first tube is a blank tube which only contains the mixture. In the second tube, 3 drops of Dragendorff’s reagent was added into the mixture, and in the third tube, 3 drops of Mayer’s reagent was added into the mixture. Formation of orange precipitates in the second tube or yellow precipitates in the third tube indicates the presence of alkaloids.

Thin Layer Chromatography
Thin Layer Chromatography (TLC) is an easy, inexpensive, and high-sensitive technique, consisting of two phases, the stationary phase and the mobile phase. Stationary phase is a thin plate of silica gel material (SiO₂) or aluminum oxide, whereas the mobile phase is a mixture of eluent solution. In this work, a mixture of n-hexane and ethyl acetate (5:1) is used as a mobile phase. Each phytochemical compound in extract will have different adsorption and solubility in the two phases. Polar compounds in the extract will be adsorbed by the stationary phase, while the non-polar compounds will move at the stationary phase layer with different rates and distances due to its polarity and solubility in the mobile phase. UV light at 254 nm and 366 nm is used for visualization of spot’s compound, and the Rf (retention factor) value of each spot’s compound can be calculated by the following formula. Rf (retention factor) = Distance traveled by sample / distance traveled by solvent

Antioxidant Activity Evaluation by DPPH Method
DPPH method was carried out to determine the antioxidant properties of Eucheuma cottonii extracts. The procedure as follows, ethylacetate extract (5 mg) and ethanol extract (5 mg) of Eucheuma cottonii were placed into a separate test tube. Amount of 10 ml of 75% ethanol was added into each of test tube to give ethylacetate and ethanol extracts of Eucheuma cottonii with the concentration of 500 μg/mL. After that, each extract of Eucheuma cottonii with the concentration of 500 μg/mL was diluted with 75% of ethanol to produce six various concentrations of 6.25, 12.5, 25, 50, 100 and 200 μg/mL of ethylacetate extract and ethanol extract, respectively. Subsequently, DPPH solution (0.1 mg/mL) was added into these six various concentrations of Eucheuma cottonii extracts. The mixture was then incubated in a dark condition at room temperature for 2 hours. The incubated mixture was then measured for its absorbance by a spectrophotometer UV-Vis at the wavelength of
The antioxidant properties is expressed as IC_{50} value. Plotting of DPPH % inhibition of sample with the concentration will produce linear line equation of Y = ax + b. The IC_{50} value of the sample could be determined by substituting Y coefficient with value of 50 to give the x value, which is equal to the IC_{50} value.

**Cytotoxic activity evaluation by MTT assay**

In this research, MTT assay is a colorimetric method that is used to measure proliferation, viability, and metabolic activity of lung A-549 cancer cells against *Eucheuma cottonii* extract.\(^\text{17}\) Extracts of *Eucheuma cottonii* were diluted until reached the concentrations of 1.56, 3.125, 6.25, 12.5, 25, 50, 100, 200 µg/mL. Extract samples with these various concentrations were added to the target cell culture of lung A-549 cancer cells. Then the cells were incubated for 48 hours at room temperature. Subsequently, 20 µl of MTT solution with a concentration of 5 mg/mL in the phosphate-buffered saline solution was added to the incubated mixture. Afterwards, the mixture were reincubated for 4 hours. Then, the mixture is centrifuged and the medium was removed. Amount of 200 µl dimethyl sulfoxide (DMSO) was added to the plate well to dissolve bluish purple sediments. The absorbance is read using at 590 nm on the microplate reader.\(^\text{19}\)

\[
\text{Inhibition Rate(%) = } 1 - \left( \frac{\text{Absorbance of Sample with Treatment}}{\text{Absorbance of Control}} \right) \times 100\%
\]

IC_{50} (50% inhibitory concentration) was calculated using Bliss assay.\(^\text{20}\)

**RESULTS AND DISCUSSION**

**Phytochemical profile of eucheumacottonii extracts**

Table 1 summarizes the phytochemical profile of *Eucheuma cottonii* extracts, which shows that all three type of *Eucheuma cottonii* extracts containmetabolites of triterpenoids and alkaloids. Triterpenoids and alkaloids are compounds that are widely distributed in plants and marine algae. Triterpenoids are organic compounds that have a variety of structure. Some triterpenoid compounds are ursolic acid, oleanolic acid, betulinic acid, celastrol, and lupeol. These triterpenoid compounds have been shown anti-tumor and anti-inflammatory properties. Triterpenoids exhibited antitumor activity by inducing apoptosis, inhibiting NF-κB activation, inhibiting cell signal transduction pathways and angiogenesis.\(^\text{18}\) Meanwhile, alkaloids are compounds bearing nitrogen atoms that have many biological activities, such as anti-asthma, analgesic effects, anti-cancer, antiproliferation and antimetastatic. Alkaloid compounds are indeed proven to be anticancer effects and have been developed into chemotherapy drugs such as camptothecin (topoisomerase I inhibitors) and vinblastine (interactions with tubulin).\(^\text{19}\)

**Thin layer chromatography (TLC) analysis of Eucheumacottonii extract**

Appearance of TLC analysis of *Eucheuma cottonii* extracts is displayed in Figure 2, whereas retention factors of phytochemical components containing in each extract is summarized in Table 2. TLC analysis shows that ethanol extract of *Eucheuma cottonii* has 2 spots of phytochemical compounds, ethyl acetate extract has 3 spots of phytochemical compounds, and n-hexane extract has only 1 spot of phytochemical compound. It seems that polar extractof ethanol and semi-polar extract of ethylacetate have similar phytochemical compounds at Rf of 0.54 and 0.84. It happened because ethanol extracts tend to contain polar chemical compounds and ethyl acetate extracts tend to contain semipolar compounds. Meanwhile, non-polar extract of n-hexane contains only one non-polar chemical compounds at Rf of 0.72, confirming that it is not similar with phytochemical compound containing in ethanol and ethylacetate extract.

**Antioxidant activity of Eucheuma cottonii extracts**

Table 3 displays antioxidant activity of *Eucheuma cottonii* extracts and ascorbic acid (as a positive control) on DPPH free radical, which is expressed as IC_{50} value. Antioxidant activity of the test sample on DPPH can be categorized based on the IC_{50} value, as follows: IC_{50} value ≤ 100 µg/mL is assigned as an active antioxidant, whereas inactive antioxidant if IC_{50} value ≥ 200 µg/mL is assigned as inactive antioxidant.\(^\text{21}\) Ascorbic acid has very active antioxidant activity on DPPH with IC_{50} value of 6.46 µg/mL. IC_{50} value for the ethanol extract was 559.76µg/mL.

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**Table 1: Phytochemical profile of Eucheuma cottonii.**

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Extract</th>
<th>n-Hexane</th>
<th>Ethylacetate</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Triterpenoid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Steroid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tannin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycoside</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 2: Retention factor (Rf) of phytochemical components in Eucheuma cottonii extracts.**

<table>
<thead>
<tr>
<th>Extract</th>
<th>Rf Value</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>0.54</td>
<td>0.84</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>0.54</td>
<td>0.84</td>
<td>0.93</td>
<td>-</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>0.72</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Rf=Retention factor*
and ethylacetate extract was 41482.81μg/mL on DPPH free radical. Therefore, both extracts did not have antioxidant activity on DPPH free radical. It seems that antioxidant activity of Eucheuma cottonii depend on the solvent used for extraction. Ethanol as polar solvent has more active as an antioxidant than ethylacetate as semi-polar solvent, because ethanol can dissolve more polar compound such as polyphenolic which possess antioxidant or free radical scavenging activity.20

Cytotoxic effect of Eucheuma cottonii extracts against lung A-549 cancer cells

Figure 3 shows the relationship between concentration of Eucheuma cottonii extract and its percentage of inhibition against lung A-549 cancer cells. In general, the percentage of inhibition against lung A-549 cancer cells increases by increasing the concentration of Eucheuma cottonii extract (concentration-dependent). Eucheuma cottonii extracts inhibit the growth of lung A-549 cancer cells at the concentration of 3.125 μg/mL and reach a maximum inhibition (50.7%) at the concentration of 200 μg/mL.

Table 4 displays cytotoxic effect of Eucheuma cottonii extracts against lung A-549 cancer cells, expressed in IC50 value, which is the median inhibitory concentration of the extract that causes 50% inhibition of lung cancer cell growth. The smaller IC50 value, the greater cytotoxic effect of the extract against lung A-549 cells. In this research, IC50 value (x) was obtained by substituting y in linear regression equation of y=ax+b with value of 50. Linear regression equation was generated by plotting of concentration of the extract in x axis with absorbance value in y axis. According to Atjanasuppat et. al., the level of cytotoxic effect of the tested extract is classified based on IC50 values, in which, it can be divided into 4 classes, as follows: IC50 value ≤20 μg/mL is classified to have a strong cytotoxic effect; IC50 value ranging from 20 to 100 μg/mL is classified to have a moderate cytotoxic effect; IC50 value ranging from 100 to 1000 μg/mL is classified to have a weak cytotoxic effect; IC50 value over 1000 μg/mL is classified to have no cytotoxic effect.21

As shown in Table 4, cisplatin as positive control with IC50 value of 3.61 μg/mL has the strongest cytotoxic effect on lung A-549 cells. Ethanol and ethylacetate extracts of Eucheuma cottonii exhibited cytotoxic effect on lung A-549 cells with IC50 values are 251.73 μg/mL and 261.41 μg/mL, respectively. In contrast with ethanol and ethylacetate extracts, n-hexane extract of Eucheuma cottonii with IC50 value of 3508 μg/mL, did not showed cytotoxic effect against lung A-549 cancer cells. This result indicating that semi-polar and polar compounds in ethylacetate and etanol extracts of Eucheuma cottonii are more effective as an anti-lung cancer on A-549 cells compared to non-polar compounds in n-hexane extract of Eucheuma cottonii.

CONCLUSION

Marine red macroalgae Eucheuma cottonii demonstrated cytotoxic effect against lung A-549 cancer cells with IC50 value of 251.73 μg/mL for ethanol extract and 261.41 μg/mL for ethanol extract. Thus, ethylacetate and ethanol extracts of Eucheuma cottonii are potential to be further developed as an anti-lung cancer agent.

ACKNOWLEDGEMENT

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest.

ABBREVIATIONS

TLC: Thin layer chromatography; DPPH: (2,2-diphenyl-1-picrylhydrazly); MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); IC50: median Inhibitory Concentration; μg/mL: microgram/millilitr; g: gram; mL: millimeter; N: Normality; UV-Vis: Ultra violet-Visible; RPMI: Rosewell Park Memorial Institute; h: hour; PBS: Phosphate-Buffered Saline; °C: degree Celsius; μL: microliter; CO2: Carbon dioxide; RF: Retention factor; USA: United States of America.

REFERENCES


Table 3: Antioxidant activity of Eucheuma cottonii extracts and ascorbic acid on DPPH.

<table>
<thead>
<tr>
<th>Tested Sample</th>
<th>IC50 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid (positive control)</td>
<td>6.46</td>
</tr>
<tr>
<td>Ethylacetate extract of E. cottonii</td>
<td>41482.81</td>
</tr>
<tr>
<td>Ethanol extract of E. cottonii</td>
<td>559.76</td>
</tr>
</tbody>
</table>

Table 4: Cytotoxic effect of Eucheuma cottonii extracts and cisplatin against lung A-549 cells.

<table>
<thead>
<tr>
<th>Extract</th>
<th>IC50 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>251.73</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>261.41</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>3508</td>
</tr>
<tr>
<td>Cisplatin (positive control)</td>
<td>3.61</td>
</tr>
</tbody>
</table>
SUMMARY

Red algae *Eucheuma cottonii* from Sorong beach, West Papua Province, bearing three phytochemical compounds. Ethanol and ethylacetate extracts of *Eucheuma cottonii* have no antioxidant activity on DPPH free radical. In contrast to antioxidant activity, both ethanol and ethylacetate extracts of *Eucheuma cottonii* showed cytotoxic effect against lung A-549 cancer cells with IC\(_{50}\) value of 251.73 μg/mL and 261.41 μg/mL, respectively.

ABOUT AUTHORS

**Dr. Ade Arsianti**: Lecture and Researcher at Medical Chemistry and Drug Development Research Center, Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia. Research interest in medicinal chemistry, synthetic organic chemistry, marine and herbal natural product chemistry.

**Gerry Kurniawan**: Medical Student, Faculty of Medicine, Universitas Indonesia. Research interest in cardiology, pulmonology, and oncology.
Nadzila Anindya Tejaputri: Medical Student, Faculty of Medicine, Universitas Indonesia. Research interest in herbal medicine, cancer biology, pediatric disease, and mental health science.

Fona Qorina: Medical Student, Faculty of Medicine, Universitas Indonesia. Research interest in herbal medicine, cancer biology, cardiovascular and metabolic disease.

Qotrunnada Fithrotunnisa: Medical Student, Faculty of Medicine, Universitas Indonesia. Research interest in herbal medicine and cancer biology.

Norma Nur Azizah, S.Si: Researcher at Drug Development Research Center, Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia. Research interest in tissue culture, analytical chemistry, and natural product chemistry in drug development.

Ajeng Megawati Fajrin: Researcher at Department of Medical Chemistry, Faculty of Medicine, Universitas Indonesia. Research interest in tissue culture, analytical chemistry, and natural product chemistry.