ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF GREEN SEAWEED ULVA LACTUCA TOWARDS MCF-7 BREAST CANCER CELLS

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Abstract
Systematic researches for breast cancer are ran in Indonesia with its flora resources and therapeutic potentials as prospective towards cancer cells through studies concerning phytochemical contents, antioxidant activity, and cytotoxicity. The aim of this research is to obtain Ulva lactuca abundant potential that is available in Indonesia as antioxidant and anticancer towards breast cancer cell MCF-7. Ulva lactuca is extracted by maceration with solvents which are n-hexane, ethyl-acetate, and ethanol, ensuing extracts of n-hexane, ethyl-acetate and ethanol. The extracts later collected and tested through phytochemical and TLC analysis. The DPPH assay determines the extracts’ antioxidant activity as a radical scavenger and MTT assay evaluates the cytotoxicity towards MCF-7 cells. Phytochemical screening revealed secondary metabolites triterpenoids, steroids, and glycosides in Ulva lactuca. The TLC resulted that there are 11 phytochemical components. From DPPH assay, ethanol and ethyl acetate extracts of Ulva lactuca are classified to have very active antioxidant activity against DPPH free radicals, with IC50 of 3.514 µg/mL and 33.770 µg/mL, respectively. From MTT assay, n-hexane extract gives IC50 value of 70.496 µg/mL, and ethyl-acetate extract gives IC50 value of 50.883 µg/mL towards MCF-7 cells, both have moderate anticancer activity. Whereas the ethanol extract gives IC50 value of 11.920 µg/mL on MCF-7 cells which considered to have active anticancer activity. Ethanol extract of Ulva lactuca that has a strong antioxidant and cytotoxic activity against MCF-7 cells should be further develop as an anti-breast cancer agent.

Keyword: Ulva lactuca, breast MCF-7 cells, cytotoxicity, antioxidant, phytochemical screening

Introduction
Cancer, more specifically breast cancer is one of the deadliest disease that affect women in Indonesia. According to GLOBOCAN evaluations in 2018, breast cancer plays specific role in increasing a high mortality rate of women. The incidence of breast cancer can occur on male, but the rate is lower in comparison to breast cancer on female.1 People
fear the involvement of free radical exposure with complex mechanism in the molecular level of cancer which result in the symptoms and clinical manifestations. Breast cancer is marked with the increasing amount of tumor molecularly from breast epithelial cells and can spread and also metastasize. Since breast cancer cases is considered as a burden among females, both psychologically and economically, hence why systematic researches to find the cure aiming to many types of cancer are conducted and have been developed ever since, including breast cancer.2

Indonesia is blessed as a country to have the expansive number of resources and diversity of flora that may have therapeutic potentials. There are lots of promising flora such as herbal and fungi that allow us to devise the prospective towards cancel cells through studies concerning phytochemical contents, antioxidant activity, and cytotoxic effect.3 Prevention of breast cancer by using herbal material is already on development for a few years even until today. This is because of its minimum to light side effects and decent effectivity as an anticancer agent.4

Phytochemical contents, antioxidant activity and cytotoxicity of an herbal extract towards cancer cell need to be known to measure the outcome effectivity, both as a preventive measure or curative measure of a cancer. It has been cleared that breast cancer is not a cancer representing a single disease, but more to the quantity of molecularly-dissimilar tumors raised from breast epithelial cells.4 Exploring Ulva lactuca green seaweed as the independent variable is solely due to its promising properties of antioxidants. There are already studies that have proven that Ulva lactuca green seaweed has the capability as herbal medicine. Nevertheless, investigations towards the use of Ulva lactuca as anticancer medication up until now are still minimum, in contrast to the availability of Ulva lactuca spread in Indonesia. This is the biggest motivation as to why we really need to do further analysis to benefit from it.5 The aim of this research is to obtain green seaweed Ulva lactuca abundant potential that is available in Indonesia waters as antioxidant and breast anticancer towards breast cancer cell MCF-7.

In this study, phytochemical contents of green seaweed Ulva lactuca is analyzed through phytochemical test, followed by antioxidant activity test with DPPH method, cytotoxic activity determination, and also examining cytotoxic activity towards breast cancer cell MCF-7 through MTT method. In these tests, using more than one type extract of Ulva lactuca aim to prove the possibilities of better activity in one extract than the other. Studies regarding green seaweed Ulva lactuca towards the medical aspect are not fully known and clear, but there have been several studies reporting about its role on several cancer cells, therefore its potential of being developed to be an anticancer agent.6

Breast cancer being the most frequent and invasive cancer in Indonesia makes it a priority to decrease the prevalence and to prevent the development of being invasive. The treatments that are presented today are commonly not accessible for most people to reach. Many inhibiting factors arise whether it is from the price of the treatments that are offered or the development of other illnesses known as the side effects. Numerous researches are being explored to look for more possibilities of treatments that give less adverse effects and affordable for the patient so they would not feel reluctant in seeking for medication. Extracting from natural vegetation may has the possibility to contribute and gain benefit for breast cancer therapy. Traditional medications from wild plants are
always been sought for novel treatments and some plants are still in need to be scientifically studied to know the medicinal purposes in them. Consequently, this research wishes to look for breast cancer treatment with the extract of Ulva lactuca

METHODS

Antioxidant Activity Evaluation by DPPH Method:

DPPH assay is used to evaluate antioxidant prospective of a compound, in this research we use Ulva lactuca extract. The concentration of DPPH depends on the source and potency of the scavenging compound. In short, a prime solution is made by 0.1 mM DPPH dissolved in ethanol. 1 mL of the solution is added to 3 mL of different extracts in ethanol at different concentration (31.25, 62.5, 125, 250, 500 μg/mL), each in different test tubes. Shake the mixture vigorously, usually with a vortex, and incubate for 30 minutes. The mixture absorbancy will be measured with spectrophotometry in 517 nm, referencing to BHT (butylated hydroxytoluene) using UV-Vis spectrophotometer. The reference we use is ascorbic acid. Most of the time, low absorbance values of reaction mixtures imply high free radical scavenging activity, but confidently free radical scavenging activity depends on the extract’s concentration. The DPPH % inhibition is used to produce linear equation, which will result in the value of IC50 calculated with the formula:

\[ \text{DPPH Neutralisation Percentage (\%) = } \left[ 1 - \left( \frac{\text{sample absorbance}}{\text{absorbance blank}} \right) \right] \times 100\% \]

Cytotoxic Activity Evaluation by MTT Assay:

Preparation of MTT assay includes dissolving, filters, sterilizing, and storing the reagent solution to make a stable solution. The tetrazolium salt MTT (3-(4,5 dimethylthiazol-2,5-diphenyltetrazolium bromide) will be reduced by an NADPH-dependent cellular enzyme which is oxidoreductase, into an insoluble formazan product that shows a purple color. The optical density will be obtained from the color observed. The MTT will require organic solvents like DMSO. To measure the optical density, we will use a spectrophotometer with a fixed wavelength filter. The absorbancy will be measured at 570 nm. Formazan solution optical density will be put on 96-well plates or cuvettes. MCF-7 cancer cells will be seeded in each well of 96-well multiplates in different concentration (increasing range of 5-500 µl). Then it will be dissolved in Dulbecco’s Phosphate Buffered Saline (DPBS), pH of 7.4 until the concentration touches 5 mg/ml. Filtration and sterilization process is done through 0.2 µM filter and moved to light and sterile container. The solution will then be stored for 4 hours at 37 °C situation to be incubated. Addition of 200 µL of DMSO into each well is done next and proceed to solubilize with a shaker, the purple color will be dissolved. The inhibition rate of cancer cell growth was plotted against the extract concentration logarithm which will produce a linear equation, determining the IC50 value. It is calculated with the formula as below:

\[ \text{Inhibition rate (\%) = } \left[ 1 - \left( \frac{\text{sample absorbance}}{\text{absorbance of control}} \right) \right] \times 100\% \]

Results and Discussion

RESULTS
Phytochemical Analysis Ulva lactuca:

Phytochemical tests were done more than once to compare the result, in which from all the test show relatively same results. Summarized by the result in the table below (see table 1), the phytochemicals that were tested are saponins, flavonoids, tanins, glycosides, triterpenoids, steroids and alkaloids. N-hexane extract of Ulva lactuca extract that are diluted in N-hexane (HE) had positive result for glycosides and triterpenoids. The ethylacetate (EA) showed positive result for glycosides and steroids. Meanwhile the ethanol (EE) only showed positive result for steroids.

Table 1. Phytochemical screening result of Ulva lactuca extract

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>N-hexane</th>
<th>Ethyl-acetate</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponins</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tanins</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Steroids</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Thin Layer Chromatography Analysis of Ulva lactuca:

TLC where the number of dots signify how many compounds present. The fluorescence glow of well-defined spots is seen under UV light. The TLC result is represented by Retention factors (Rf) value. The Rf value is identified by comparing the distances that the sample has traveled to the origin distance traveled by solvent. The figure showed us the principle of TLC with the measured solvents being n-hexane, ethyl-acetate, and ethanol, respectively. The silica gel being a stationary phase that is polar, making it possible for the polar extract to showed the retention factors value that is less than 0.05. From the result of the TLC in the figure on the side (see figure 2), mixture of n-hexane and ethyl-acetate with the ratio of 4:1 is used as mobile phase and the silica gel is used as the stationary phase. From the calculation appeared on table 2, n-hexane extract has the most phytochemical components (five components) with Rf value of 0.5, 0.6, 0.8, 0.8 and 0.9 respectively. Ethanol extract has the least phytochemical components (two components) with Rf value of 0.2 and 0.5.
Figure 2. TLC result of Ulva lactuca extract

Table 2. Rf value and phytochemical components detected from Ulva lactuca extract

<table>
<thead>
<tr>
<th>Phytochemical components detected</th>
<th>N-hexane</th>
<th>Ethyl acetate</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rf value</td>
<td>0.5, 0.6, 0.8, 0.8, and 0.9</td>
<td>0.6, 0.8, 0.9, an 0.9</td>
<td>0.2 and 0.5</td>
</tr>
<tr>
<td>The number of components detected</td>
<td>5</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

Antioxidant Activity of Ulva lactuca:

Antioxidant activity of Ulva lactuca extract is evaluated through DPPH assay. Antioxidant activity of the extract is presented as the IC50 value. Antioxidant activities were tested only for ethyl acetate extract and ethanol extract, due to n-hexane extract being a non-polar extract which not dissolved well in ethanol solvent. The tests were done multiple times and the chosen result are as below (see table 3).

Table 3. IC50 of DPPH assay

<table>
<thead>
<tr>
<th>Extract</th>
<th>IC50 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>0.69</td>
</tr>
<tr>
<td>Ethyl-acetate</td>
<td>33.77</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3.51</td>
</tr>
</tbody>
</table>

The inhibition percentage of extract against the DPPH free radicals will be made into the line-graph of concentration in x axis versus percent inhibition in y axis to generate a straight-line equation of \( y = ax + b \), where the inhibition percentage is the y axis and log extract concentration being the x axis.

The IC50 value will be determined by the scavenging rate of ascorbic acid, ethyl acetate and ethanol. The IC50 of ascorbic acid will work as positive control to be compared with the IC50 value of Ulva lactuca extract towards DPPH free radicals. The IC50 value of ethyl-acetate extract of 33.77 μg/mL is resulted by substituting \( y=50 \) on the straight-line equation of \( y = 12.283x + 31,225 \) (\( R^2 = 0.90408 \)) (see figure 3).
Figure 3. Linear graph of ethyl-acetate extract towards DPPH assay
Meanwhile the IC50 value of ethanol extract is 3,514 μg/mL. The value is the result from substituting y=50 on the equation of y = 8,2592x + 45,492 (R² = 0,97805) (see figure 4).

Figure 4. Linear graph of ethanol extract towards DPPH assay

Cytotoxic Activity of Ulva lactuca Extracts towards MCF-7 Cancer Cells:
From figure 5, the line graph shows the IC50 value of N-hexane extracts of Ulva lactuca versus the inhibition percentage against MCF-7 cancer cells (see figure 5). Figure 6 displays the line equation for the concentration of ethyl-acetate extract of Ulva lactuca versus its percentage of inhibition against MCF-7 cells (see figure 6). The line equation for the concentration of ethanol extract of Ulva lactuca versus its percentage of inhibition against MCF-7 cells can be seen from figure 7 (see figure 7).

Figure 5. Linear graph of n-hexane extract towards MTT assay
The IC50 values of extracts of Ulva lactuca against breast MCF-7 cells is summarized in Table 4 (see table 4).

Table 4. Cytotoxic Activity of Ulva lactuca extracts towards MCF-7 Breast Cancer in IC50 value

<table>
<thead>
<tr>
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<th>Mean IC50 (μg/mL)</th>
</tr>
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<tbody>
<tr>
<td>Doxorubicin</td>
<td>1.6683E-08 (±2.36E-08)</td>
</tr>
<tr>
<td>N-hexane</td>
<td>70.49 (±42,18)</td>
</tr>
<tr>
<td>Ethyl-acetate</td>
<td>50.88 (±20.48)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>11.92 (±9,61)</td>
</tr>
</tbody>
</table>
The ± value on mean result is the standard deviation (SD) from three separate experiments. All of the extracts are showing relatively low IC50 value. N-hexane extract of *Ulva lactuca* shows IC50 value of 70.496 μg/mL, ethyl-acetate extract shows IC50 value of 50.883 μg/mL, and ethanol extract shows IC50 value of 11.919 μg/mL. Doxorubicin as the positive control shows the IC50 value of 1.6683E-08 μg/mL. From the statistical analysis, we can relate that the data distribution is not normal due to abnormal distribution of n-hexane (p < 0.05) (see table 5).

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<table>
<thead>
<tr>
<th>Shapiro-Wilk Statistic</th>
<th>df</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.767</td>
<td>3</td>
<td>.038</td>
</tr>
<tr>
<td>0.901</td>
<td>3</td>
<td>.390</td>
</tr>
<tr>
<td>0.862</td>
<td>3</td>
<td>.273</td>
</tr>
<tr>
<td>0.750</td>
<td>3</td>
<td>.000</td>
</tr>
</tbody>
</table>

Therefore the Kruskal-Wallis test is used. From the 6), the test result is value between different $\chi^2(2) = 8.744$, p = suggestion of a statistically significant result. The IC50 values mean rank are 9.67 (n-hexane), 5.33 (ethanol), and 9.00 (ethyl-acetate).

### Table 6. Kruskal-Wallis non-parametric test

<table>
<thead>
<tr>
<th>Kruskal-Wallis H</th>
<th>IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df</td>
<td>3</td>
</tr>
<tr>
<td>Asymp. Sig.</td>
<td>.033</td>
</tr>
</tbody>
</table>

**DISCUSSION**

**Phytochemical Constituents of *Ulva lactuca***:

Green algae being the most diverse algae allows human to do species exploration, including *Ulva lactuca*. The secondary metabolites that had been derived are triterpenoids, tannins, steroids, amino acids and phenolic compounds.\(^{51}\)

The phenolic compounds are known as antioxidant that have positive interest towards preventing and curing many diseases.\(^ {52}\) Whereas triterpenoids and steroids are proved from several studies to possess cytotoxicity and radical scavenging activity, respectively. Triterpenoids are capable of inducing apoptosis of cancer cells with no threat of harm to the normal cells.\(^ {53}\) A study by Sahidin et al mentioned that triterpene steroids stigmasterol compound showed moderate antioxidant activity.\(^ {54}\) From preliminary qualitative screening by Ade Arsianti et al in 2016, it is found that flavonoids, tannins, glycosides, and steroids were present for *Ulva lactuca*.\(^ {55}\) However, in this research, the metabolites that showed positive result were glycosides, triterpenoids, and steroids. Ethanol extract of *Ulva lactuca* showed no positive reaction except to steroids, indicating that steroid has greater intensity than other metabolites. Similar result showed that all extracts prove positive result of glycosides except for ethanol extract. Phytochemical constituents contained in *Ulva lactuca* are showed to be negative due to the possibility of being in small amounts. The metabolites differences are possible due to various degree
in the habitat, but can be concluded that *Ulva lactuca* is a respectable source of phytochemical.

The TLC analysis resulted in the Rf value between three extracts of *Ulva lactuca*. Based on TLC analysis of these extracts, there are 11 number of phytochemical components in total. The noticeable extract is n-hexane extract, that has 5 components, in comparison to ethyl acetate extract that has 4 components and ethanol extract that only has 2 components. From the TLC analysis between the comparison of three extracts, it is implied that there are compounds present in *Ulva lactuca* extracts that are showing the same Rf value. One, n-hexane extract being compared to ethanol extract showing the same Rf value of 0.5. Also, n-hexane extract being compared to ethyl-acetate extract showing the same Rf value of 0.6, 0.8 and 0.9.

**Antioxidant Activity of *Ulva lactuca*:**

The phytochemical constituents containing in the extract sample play roles that influence antioxidant activity and cytotoxic activity. DPPH assay is used to measure the radical scavenging activity of the tested sample, with the result interpreted as IC50 which is the needed sample concentration to inhibit half (50%) of the DPPH free radical. The ascorbic acid is the reference compound to compare the result of IC50. We did not consider n-hexane extract due to it being a non-polar solvent and it is not miscible in ethanol solvent.

Ascorbic acid as a positive control showed the most effective antioxidant activity on DPPH free radical with IC50 value of 0.69 μg/mL. Ethanol extract of *Ulva lactuca* has IC50 value of 3.514 μg/mL on DPPH free radical, whereas ethyl acetate extract has 33.770 μg/mL. According to Marjoni et al in 2017, antioxidant classification, both ethanol and ethyl acetate extracts of *Ulva lactuca* with IC50 value <50 μg/mL, were classified to have very active antioxidant activity (<50 μg/mL). The *Ulva* species high activity result may be caused by their phenolic compounds within them.

In comparison to the study ran by Eka Prasedya et al, who took *Ulva lactuca* seaweed from the coastal locations of Lombok Island, the ethanol extract of *Ulva lactuca* was considered to have lower IC50 value classified as moderate (534.76 ± 14 μg/mL). The difference result between these studies is influenced by possible environmental factors in different places, such as the exposure to UV lights during low tides. Therefore, it could alter the phytochemical productions of the seaweeds, led to influenced antioxidant activity.

**Cytotoxic Activity of *Ulva lactuca* Extracts towards Breast Cancer Cell Line MCF-7:**

In this study, the effect of *Ulva lactuca* extract in different solvents towards MCF-7 cell proliferation was explored. The cancer cell was exposed to *Ulva lactuca* extracts for 24 hour and checked by MTT assay. Since we already knew from the preliminary phytochemical screening that triterpenoids and steroids possess radical scavenging and cytotoxic activity, and both metabolites are contained in the *Ulva lactuca* extract, therefore we can continue the exploration by doing MTT assay. MTT assay is used to evaluate the cytotoxicity of substances, which is interpreted as IC50. Cell growth is supposed to be inhibited in a time and concentration-dependent manner. The cytotoxic
activity of each extracts of *Ulva lactuca* shows satisfying IC50 values. Atjanasuppat et al in 2009 classified anticancer activity based on IC50 value in which <20 μg/mL indicates an active anticancer activity, 20-100 μg/mL indicates a moderate anticancer activity, 100-1000 μg/mL indicates a weak anticancer activity, and >1000 μg/mL indicates a non-active anticancer activity. Based on the MTT assay result, n-hexane extract of *Ulva lactuca* gives IC50 value of 70.49589 μg/mL and ethyl-acetate extract of *Ulva lactuca* gives IC50 value of 50.88308 μg/mL against MCF-7 cells. Both of the extracts (IC50 value of 20-100 μg/mL) belong to moderate cytotoxicity, whereas ethanol extract of *Ulva lactuca* gives IC50 value of 11.91971 μg/mL against MCF-7 cells, which belongs to active or strong cytotoxicity (<20 μg/mL) classification. The positive control of doxorubicin exhibits IC50 value of 2.3593E-08 meaning the doxorubicin has very active anticancer activity on MCF-7 cells.

Similar study done by Sakinah Mashjoor et al in 2016 on testing other Ulvaceae family (*Ulva flexuosa*) against MCF-7 cells. MTT assay was done and the result showed that ethyl-acetate extract of *Ulva flexuosa* exhibited IC50 value of 86.37 μg/mL, which belongs to moderate cytotoxic activity (20-100 μg/mL), similar to the result that we have obtained from our research.

Even though the ethyl-acetate and ethanol extract of *Ulva lactuca* have a normal distribution, the statistical analysis result is done with a non-parametric test Kruskal-Wallis instead of one-way ANOVA since one of the data, which is the data of n-hexane extract, that apparently do not have normal distribution (p < 0.05). Kruskal Wallis test showed IC50 value between different extracts to be 0.033 leading to statistically significant result.

The relation between ethanol and ethyl-acetate extract of *Ulva lactuca* is that both extracts showed satisfying results of cytotoxic activity and antioxidant activity. Both ethanol extract and ethyl-acetate extract of *Ulva lactuca* were classified to have very active antioxidant activity, whereas on the other hand ethanol has strong cytotoxic activity and ethyl-acetate has moderate cytotoxic activity.

**CONCLUSION**

The secondary metabolites contained in green seaweed *Ulva lactuca* extracts are glycosides, triterpenoids, and steroids. In total, from the TLC analysis there are 11 phytochemical components. Ethanol and ethyl-acetate extract of *Ulva lactuca* exhibit antioxidant activity towards DPPH free radical. N-hexane and ethyl-acetate extracts of *Ulva lactuca* have a moderate cytotoxicity, whereas ethanol extract of *Ulva lactuca* has a strong cytotoxic activity towards MCF-7 breast cancer cells.

**REFERENCES**


