Antioxidant activity of methanolic extract of *Eucheuma spinosum* extracted using a microwave

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**Abstract.** *Eucheuma spinosum* is a widely cultivated red seaweed. The phenolic compounds contained in *E. spinosum*, especially flavonoids, can be used as a source of natural antioxidants. This study aimed to determine the antioxidant activity of *E. spinosum* extracted using a microwave with different extraction times (6, 8 and 10 minutes). The parameters measured included yield, phytochemical test, total phenolic content, total flavonoid content and antioxidant activity, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP). The results showed that the *E. spinosum* extract with an extraction time of 10 minutes had the highest yield with a total phenolic content of 59.42 ± 27.36 mg GAE/g and a total flavonoid content of 65.48 ± 25.34 mg QE/g. The results of phytochemical screening showed that the extract contain triterpenoid compounds. The antioxidant activity of the *E. spinosum* extract showed the highest IC50 value of 46.52 ± 5.53 ppm using the DPPH method and the FRAP method was 58.92 ± 1.76 μM/g. Keywords: red seaweed, microwave extraction method, DPPH

1. Introduction

Seaweed is one of the macroalgae that has economic potential which can be used as raw material in industry and health [1]. Red seaweed was a seaweed that was widely cultivated, one of which was *Eucheuma spinosum*. This seaweed has many benefits including as a food, cosmetic and medicinal ingredient [2] because it contains flavonoids [3], so it can be used as a source of natural antioxidants [4].

The antioxidant activity of seaweed was known through several stages, one of which was extraction. According to Rifai et al. [5], the extraction of seaweed as a source of antioxidants was influenced by several factors, namely the method and length of extraction time. The extraction time used will affect the extraction yield. The longer the extraction time was used, the greater the extraction results obtained [6]. Several methods can be used in plant extraction such as maceration, percolation, reflux, and soxhlet [7]. In addition, there was also an extraction method that can be used, namely Microwave Assisted Extraction (MAE), where this method requires a shorter and more efficient extraction time [8] and was environmentally friendly [9]. Extraction using a microwave requires a smaller amount of solvent when compared to other methods such as Soxhlet, for example [10].

Several studies on antioxidant activity using the microwave method on seaweed and other plants that have been carried out include the use of the microwave method in the extraction of phenolic compounds from four species of brown macroalgae, namely *Ascophyllum nodosum, Laminaria*
japonica, Lessonia trabeculate and Lessonia nigrecens [8], optimal microwave extraction conditions for phenolic compounds and the antioxidant capacity of brown algae Sargassum vestitum [11], flavonoid content and antioxidant activity of Pleurotus ostreatus extract [6], extraction of pelawan leaves (Tristaniopsis merguensis) as antioxidants using the microwave method [7]. Research related to the effect of extraction time on the antioxidant activity of red seaweed E. spinosum using microwave was still limited. Therefore, this study aims to determine the antioxidant activity of E. spinosum seaweed which was extracted using a microwave with a variation of extraction time.

2. Materials and method

2.1. Materials

The main material used in this research was the seaweed Eucheuma spinosum taken from Bali's Nusa Penida waters. The chemicals used for extraction and analysis include methanol, H$_2$SO$_4$, HCl, Follin Ciocalteau reagent, quercetin, AlCl$_3$, 2,2-diphenil-1picrylhydrazil (DPPH), CH$_3$COONa.3H$_2$O produced by Merck, USA. FeCl$_3$.6H$_2$O, FeSO$_4$.7H$_2$O, and 2,4,6-tripyridyl-s-triazine (TPTZ) produced by Sigma-Aldrich, Germany.

2.2. Sample preparation

E. spinosum seaweed was taken from the waters of Batumulapan Village, Nusa Penida District, Klungkung Regency, Bali Province in January 2020. The collected fresh seaweed was then washed using fresh water to remove the dirt. The seaweed was then dried using a drying method according to Ling et al. (2015) [12] modified. The seaweed that has been washed was then put in the oven at 40°C for 24 hours. Samples that have been oven were then mashed using a blender and then sieved to get a uniform size, then stored in an airtight container. Samples in containers were stored in the freezer pending further analysis.

2.3. E. spinosum extraction

The method of extraction of E. spinosum seaweed using a microwave refers to the research of Yuan et al. [8] modified. The sample extraction steps taken were seaweed E. spinosum, which had been mashed, weighed 30 grams, then put it in a container then added 300 ml of 70% methanol solvent and stirred first. Samples were then extracted using a microwave using temperature variations of 6, 8, and 10 minutes with the heat intensity set to low levels. The extraction results were then filtered using filter paper and then centrifuged. Next, the supernatant was evaporated using a vacuum rotary evaporator and dried using a freeze dryer. After that, the extract was then stored at -20°C before being analyzed further.

2.4. The yield

The yield was obtained from the ratio between the weight of the extract and the weight of the sample used. The yield calculation used refers to Kurniawati et al. [13]. The yield obtained is calculated using the following formula:

\[
\text{Yield} \, (\%) = \frac{\text{Extract weight}}{\text{Sample weight}} \times 100\%
\]

2.5. Phytochemical test

Phytochemical testing conducted to identify the content of the active compound contained in a substance or plant. The phytochemical content of seaweed varies depending on the type or species [14]. Testing of phytochemical compounds refers to research conducted by Sangi et al. [15] with modifications. The phytochemical tests included steroid, triterpenoid, and tannin tests.
2.6. Total phenol
The total phenol testing method used refers to the research of Ahmad et al. [16] modified with gallic acid as standard. The *E. spinosum* seaweed extract was weighed as much as 40 mg then dissolved in 10 mL of methanol. The extract solution was then taken as much as 1 mL and 0.4 mL of the Folin-Ciocalteau reagent was added. The solution was then shaken and left for 4-8 minutes and then 4.0 mL of 7% Na₂CO₃ solution were added until homogeneous. The solution was added with aquabides up to 10 mL and then left for 2 hours at room temperature. The absorption was then measured at a wavelength of 744.8 nm. The phenol content obtained was expressed as mg of gallic acid equivalent (GAE)/g of extract. The formula for calculating the total phenol of *E. spinosum* extract [4] is as follows:

\[
\text{Total phenol (GAE/g) = } \frac{mg}{g} \cdot \frac{GAE}{c} \cdot \frac{v}{m}
\]

Note:
- \(c\): total phenol from standard curve (mg/L)
- \(v\): extract volume (L)
- \(m\): extract weight (g)

2.7. Total flavonoids
The total flavonoid test carried out refers to the method described by Ahmad et al. [16] modified with quercetin as standard. The extract of *E. spinosum* seaweed was weighed as much as 40 mg dissolved in 10 mL of methanol. Then 1 mL were taken and added 3 mL of methanol, 0.2 mL of 10% AlCl, 0.2 mL of potassium acetate, and added with aquabidestillata until the volume reached 10 mL. The solution was then stored in a dark place for 30 minutes at room temperature. The absorbance measurement used UV-Vis spectrophotometry with a wavelength of 431 nm. The levels of flavonoids obtained were expressed as quercetin equivalent. The formula for calculating the total levels of *E. spinosum* flavonoids according to Syafitri et al.[17] as follows:

\[
\text{Total flavonoid (QE) = } c \cdot \frac{V}{m}
\]

Where:
- \(c\): total flavonoid concentration from the standard catechin curve (mg/L)
- \(V\): volume of extract (L)
- \(m\): extract weight (g)

2.8. Inhibitory activity of 2,2 diphenyl-1-picrihydrazil (DPPH)
Testing antioxidant activity with DPPH inhibition refers to Handayani et al. [18] with modification. A total of 4 ml of DPPH solution was taken, then vortexed and then incubated in a dark room at 37°C. The absorbance was measured at a wavelength of 517 nm. Measurement of the antioxidant activity of the *E. spinosum* extract was carried out by taking 0.5 ml of sample solution from each concentration, then adding 3.5 ml of DPPH. The solution was vortexed, then incubated in a dark room at 37°C. The absorbance was measured at a wavelength of 517 nm. Measurement of the antioxidant activity of the standard solution by taking a quercetin solution of 0.5 ml for each concentration. Then each concentration was added to 3.5 ml of DPPH solution. The solution was vortexed, then incubated in a dark room at 37°C. The absorbance was measured at a wavelength of 517 nm. IC₅₀ calculation refers to Dewi et al. [6]. The IC₅₀ value was obtained by making a curve between the sample concentration and % inhibition of antioxidants with the formula for calculating % inhibition, namely:

\[
\text{Inhibition activity (\%) = } \frac{\text{Absorbance of blank} - \text{Absorbance of sample}}{\text{Absorbance of blank}} \times 100\%
\]
2.9. Ferric Reducing Antioxidant Power (FRAP) activity

The FRAP activity test refers to the method described by Selawa et al. [19] modified. The standard solution of FeSO\(_4\).7H\(_2\)O was prepared by taking 100 mL of a stock solution of 10,000 μmol/L FeSO\(_4\).7H\(_2\)O and then diluting it to 1,000 mL to get a concentration of 1,000 μmol/L FeSO\(_4\).7H\(_2\)O. Furthermore, a 1,000 μmol/L FeSO\(_4\).7H\(_2\)O solution was taken as much as 0.1 each; 0.2; 0.3; 0.4; and 0.5 mL and then put into a different measuring flask and diluted with 100 mL distilled water. The concentrations of the standard FeSO\(_4\).7H\(_2\)O solution formed were 1, 2, 3, 4, 5 μmol/L respectively. The solution was taken as much as 1 mL and then added 3 mL of FRAP reagent and read at each wavelength in the range 588-598 nm using a UV-Vis spectrophotometer.

A total of 10 mg of quercetin is weighed and then added with 10 mL of distilled water to form a concentration of 1,000 ppm. Then 0.1 mL was taken and methanol added to 10 mL. The quercetin standard solution was then read the absorbance using a spectrophotometer with a wavelength of 596 nm. Determination of the absorbance of the *E. spinosum* sample by taking 10 mL of *E.spinosum* extract was then added with 10 mL of methanol and 0.1 mL of the *E. spinosum* extract solution was then added with 3 mL of the FRAP reagent and put into a test tube. The solution was then read for its absorbance using a spectrophotometer with a maximum wavelength (596 nm).

2.10. Data analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS). The homogeneity test was carried out by Levene's Test while the normality test was carried out using the Kolmogorov-Smirnov Test. Parametric research data were tested using the Least Significance Different (LSD) test and Duncan's test, while non-parametric research data were tested using the Kruskal-Wallis test and the Mann-Whitney advanced test.

3. Result and discussion

3.1. The yield of *E. spinosum* extract

The yield of *E. spinosum* extract using microwave and methanol solvent for 6, 8 and 10 minutes was 3.62 ± 0.35, 4.78 ± 0.06 and 6.85 ± 0.55%, respectively. Based on Table 1 the highest yield of *E. spinosum* extract was in the extraction time of 10 minutes, namely 6.85 ± 0.55% and the lowest yield was in the extraction time of 6 minutes, namely 3.62 ± 0.35%. The yield of each treatment also tends to increase with increasing extraction time. This is due to the longer extraction time which will produce increased heat so that the pressure in the material will also increase and cause damage to the cell walls of the material and compounds will move from the material to the solvent [20]. Sasmito & Pusparani [21] stated that the longer the extraction time, the higher the yield will be because the contact between the material and the solvent was longer. The results of this study were lower than the research conducted by Sari et al. [22] on dried *E. spinosum* seaweed extracted using the maceration method (yield of 12.44%). This is due to the use of a different extraction method with a longer extraction time, namely maceration for 24 hours.

**Table 1. Effect of extraction time on the yield, total phenol, and total flavonoids of methanolic extract of *E. spinosum* extracted using microwave**

| Extraction time (minutes) | Yield (%) | Total phenol (mg GAE/g) | Total flavonoids (mg QE/g) |
|--------------------------|-----------|-------------------------|---------------------------|
| 6                        | 3.62 ± 0.35\(^a\) | 46.22 ± 5.07\(^a\)     | 103.33 ± 12.02\(^a\)     |
| 8                        | 4.78 ± 0.06\(^b\)  | 59.21 ± 3.59\(^b\)     | 106.11 ± 17.98\(^a\)     |
| 10                       | 6.85 ± 0.55\(^c\)  | 62.98 ± 6.40\(^b\)     | 109.44 ± 15.03\(^a\)     |

\(^{a}\) Different letters in the same row indicate significant differences (P <0.05)
3.2. **Phytochemical test of E. spinosum extract**

Phytochemical screening was a step used to identify a compound contained in the simplicia or plant to be tested. Based on Table 2, it shows that *E. spinosum* extract contains triterpenoid compounds but does not contain steroids and tannins. This was in accordance with research conducted by Sari et al. [22] which stated that the dry extract of *E. spinosum* did not find steroid and tannin compounds. The results showed that the extraction time had an effect on the color intensity of the *E. spinosum* extract triterpenoid test. According to Widiyati [23], this was because the resulting triterpenoid content varies from (+) a little, (++) quite a lot, (+++) a lot and (+++++) very much.

Table 2. Effect of extraction time on the phytochemicals of methanolic extract of *E. spinosum* extracted using a microwave

| Compound group | Extraction time (minutes) | Description |
|---------------|---------------------------|-------------|
| Steroids      | - | - | - | No blue color formed |
| Triterpenoids | ++ | + | + | Formed a purplish orange color |
| Tannins       | - | - | - | No dark blue / green color formed |

Note:

(+) : contained a little compound/lighter color

(++) : contains a lot of compound/more concentrated color

Muhtar et al. [24] stated that *E. spinosum* seaweed contains compounds including flavonoids, triterpenoids, alkaloids, ascorbic acid which can be used as antioxidants as well as phenol and phlorotannin compounds. Several studies have been conducted and proved the existence of triterpenoid content in *E. spinosum* seaweed, including *E. spinosum* which is extracted using ethanol [22] and methanol [3].

3.3. **Total phenolic content of *E. spinosum* extract**

The results of the total phenolic content test of *E. spinosum* extracted using methanol with the help of microwaves with different extraction times can be seen in Table 1. The total phenolic content in the *E. spinosum* extract increases with the length of extraction time. The highest total phenolic content was obtained at 10 minutes extraction time of 62.98 ± 6.40 mg GAE/g and the lowest yield was obtained at 6 minutes at 46.22 ± 5.07 mg GAE/g. This was due to the fact that the extraction time was too short, resulting in the withdrawal of phenolic compounds less optimal so that the material has not been extracted completely compared to the longer extraction time.

Research using a similar extraction method and length of time has been conducted by Sari et al. [22] on *Eucheuma cottonii* seaweed and obtained the best total phenolic content at 6 minutes extraction time with a temperature of 60°C and an additional time of above 8 minutes at the same temperature did not increase the total phenolic content. The difference was due to the use of different extraction temperatures. Increasing the temperature can increase or decrease the extracted phenolic compounds. The increase in temperature causes the content of phenolic compounds to increase to a certain temperature but can cause a decrease in the content of phenolic compounds due to the decomposition of phenolic compounds and produce new components that were lower than the boiling point so that they were more volatile [22].

3.4. **Total flavonoid content of *E. spinosum* extract**

The results of testing the total flavonoid content in *E. spinosum* extract with extraction times of 6, 8 and 10 minutes, were 103.33 ± 12.02, 106.11 ± 17.98 and 109.44 ± 15.03 mg QE/g, respectively (Table 1). The highest total flavonoid content was obtained at the extraction time of 10 minutes, while the lowest yield was obtained at 6 minutes. The difference in results can be caused by variations in the extraction time used. According to Dewi et al. [6], the longer the extraction time, the longer the microwave exposure to the sample, which causes the interaction of flavonoids with solvents to be
more effective and the more flavonoids extracted will be. MAE was a method that utilizes energy from microwaves that create heat and cause temperatures to rise. Heating with microwaves causes the cell wall of the material to be damaged so that the target compound will be easily attracted and dissolved in the solvent [25].

The use of varying extraction times did not significantly affect the total flavonoid content of E.spinosum extract. Research with similar methods and treatments has been reported by Kristanti et al. [26] on the Zea mays L. extract using MAE with an extraction time of 18 minutes, the total flavonoid content was 149 mg QE/g extract. The total flavonoid content in the onion peel extract Allium cepa L. which was studied by Setiani et al. [27] extracted using MAE for 6 minutes and the result was 17.18%.

3.5. Antioxidant activity of E.spinosa using the DPPH method

The IC$_{50}$ value of antioxidant activity using the DPPH method in E. spinosa extract can be seen in Table 3. The IC$_{50}$ value for quercetin was 14.16 ± 0.78 ppm, while the IC$_{50}$ value in the E. spinosa extract with extraction time 6, 8 and 10 minutes was 38.82 ± 7.09, 37.76 ± 5.43 and 36.30 ± 9.20 ppm, respectively. The lower the IC$_{50}$ value, the higher the antioxidant activity of the material being tested [6]. Table 3 shows that the highest antioxidant activity was obtained in the E. spinosa extract with an extraction time of 10 minutes, namely 36.30 ± 9.20 ppm and the lowest antioxidant activity was obtained in the extraction time of 6 minutes at 38.82 ± 7.09 ppm. This can be influenced by the content of phenolic and flavonoid compounds found in E. spinosa extract.

| Sample         | IC$_{50}$ (ppm)      |
|----------------|---------------------|
| Quercetin      | 14.16 ± 0.78        |
| E.spinosa (6 minutes) | 38.82 ± 7.09      |
| E.spinosa (8 minutes) | 37.76 ± 5.43    |
| E.spinosa (10 minutes) | 36.30 ± 9.20    |

*the same letter in the same row indicates a significant difference (P <0.05)

The higher the phenolic and flavonoid content in a material, the higher the antioxidant activity, so that the IC$_{50}$ value obtained is lower. According to Dewi et al. [6], to stabilize free radical compounds, flavonoids will donate hydrogen or electrons to radical compounds so that the higher the flavonoid content, the antioxidant activity will increase. Research related to the antioxidant activity of dry E. spinosa has been reported by Sari et al. (2015) [22] with IC$_{50}$ results of 472.14 ppm using the maceration method. The results of the antioxidant activity were much lower when compared to the IC$_{50}$ value of E. spinosa extracted using the MAE method. This can happen because in the MAE method the use of microwaves can reduce the occurrence of enzymatic activity which can cause damage to the active compound to be extracted so that more compounds are obtained in a shorter time [6].

3.6. The antioxidant activity of E.spinosa using the FRAP method

The results of the antioxidant activity test using the FRAP method are expressed in terms of Fe (II) equivalence, which was a solution that produces the same absorbance as 1 mM FeSO$_4$ solution. Table 4 shows that the highest FRAP value of the E. spinosa extract was obtained at the extraction time of 10 minutes, namely 59.53 ± 1.40 µM/g and the lowest value at 6 minutes of 61.47 ± 1.40 µM/g. According to Yefrida et al. [28], the more the amount of Fe$^{3+}$ TPTZ that was reduced to Fe$^{2+}$ by the sample, the greater the antioxidant activity of the sample. Fe$^{3+}$ TPTZ is considered as an oxidizing compound that may be present in the body which has the potential to cause damage to body cells, while the sample extract is the antioxidant.
Based on statistical testing, it is known that there was no significant difference (P > 0.05) to the FRAP value of each sample. The use of different antioxidant activity test methods can provide various results due to the influence of the chemical structure of antioxidants, sources of free radicals to the physico-chemical properties of different sample preparations [29]. Fe$^{3+}$ in the FRAP test comes from the FRAP reagent, namely a mixture of acetate buffer, TPTZ and FeCl$_3$. When Fe$^{3+}$ was reduced to Fe$^{2+}$ it will form a blue color in the test solution. If the blue color becomes darker, the more Fe$^{2+}$ ions will be formed. The darker the blue color intensity, the higher the potential for antioxidant activity of a sample [30].

Table 4. Effect of extraction time on the FRAP value of quercetin and methanolic extract of E. spinosum extracted using a microwave

| Sample                  | Concentration (ppm) | FRAP (μM/g) |
|-------------------------|---------------------|-------------|
| Quercetin               | 10                  | 54.00 ± 1.91|
| E. spinosum (6 minutes) | 1000                | 61.47 ± 1.40*|
| E. spinosum (8 minutes) | 1000                | 60.53 ± 5.46*|
| E. spinosum (10 minutes)| 1000                | 59.53 ± 1.40*|

*the same letter in the same row indicates a significant difference (P < 0.05)

4. Conclusion
The antioxidant activity of the E. spinosum extract increased tend to along with the extraction time. There was correlation between total phenolic and flavonoid content in the material with antioxidant activity. The best results were obtained in the E. spinosum extract with an extraction time of 10 minutes with an IC$_{50}$ value of 36.30 ± 9.20 and a FRAP value of 59.53 ± 1.40 μM/g.

Acknowledgments
The author would like to thank the Faculty of Agriculture, Universitas Gadjah Mada for funding this research through the 2020 Faculty of Agriculture UGM Lecturer and Student Collaboration Research Grant scheme with contract number 1508/PN/PT/2020. This paper is part of the first author's Bachelor Thesis.

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