Antioxidant Activity and Tyrosinase Inhibition of Red Seaweed (*Kappaphycus alvarezii*) Extracted Using Different Solvents

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Abstract. The common type of seaweed in Indonesia is *Kappaphycus alvarezii*. Since this seaweed thrives in tropical waters, antioxidants are suspected to protect it from the adverse effects of UV radiation. Studies also suggest that extracts from this seaweed can also protect skin from damage caused by UV exposure by inhibiting tyrosinase activity. This study aimed to determine the antioxidant activity and tyrosinase inhibition of *K. alvarezii* extracted using the maceration method with three different solvents: 70% ethanol, ethyl acetate, and n-hexane. *K. alvarezii* extracts are active in the antioxidant mechanism through electron transfer, shown by the result of the FRAP antioxidant capacity of ethyl acetate extract (36,550±0,127 µmol TE/g CE). The 70% ethanol extract demonstrated the highest DPPH antioxidant capacity (9,785±0,084 µmol TE/g CE) and showed no significant difference with ethyl acetate extract (9,540±0175 µmol TE/g CE). The 70% ethanol extract also exhibited the highest inhibition of lipid peroxidation (88,663±0,246%) at 10 mg/mL. All extracts showed tyrosinase inhibition but to a lesser extent than that of the positive control kojic acid. The inhibition percentages at 10 mg/mL were respectively 70% ethanol extract (43,016±3,064%), ethyl acetate extract (41,474±1,365%), and n-hexane extract (37,515±0,467%). This study concluded that *K. alvarezii* extracts can be used as an antioxidant and tyrosinase inhibitor when extracted using ethanol or ethyl acetate.

1 Introduction

Indonesia is known to be the world's largest producer of red seaweed *Kappaphycus*. Its production represents 65% of the global *Kappaphycus*. High cultivation activity in Indonesia was supported by the ease of cultivation, harvesting, drying techniques, low initial capital investment, and a short production cycle [1].

The abundant *Kappaphycus* species in Indonesia is *Kappaphycus alvarezii*. Its ability to thrive in tropical waters is thought to be due to the presence of antioxidant compounds that

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protect against the adverse effects of sunlight radiation. *K. alvarezii* from Sulawesi extracted with ethanol has been shown to have high antioxidant activity against DPPH, with an IC_{50} of 30,036 ppm [2]. FRAP method testing showed the antioxidant capacity of *K. alvarezii* ethanol extract of 253,11 mg PGE/g [3]. However, there has been no study of *K. alvarezii* extract to investigate the antioxidant activity in inhibiting lipid peroxidation using the TBA-MDA method, which is considered a good indicator of oxidative stress in biological systems [4].

Antioxidant activity can also be used to protect the skin from UV exposure. Dolorosa *et al*. [5] showed that the methanol extract of *K. alvarezii* has an IC_{50} against tyrosinase of 2.691,48 µg/mL. However, methanol can be toxic if exposed through consumption, skin absorption, and inhalation. Based on this, the use of other safer solvents needs to be explored.

Currently, commonly used commercial ingredients for hyperpigmentation are hydroquinone and kojic acid. Although hydroquinone has a strong inhibitory effect on tyrosinase, its penetration into the skin is poor and there is a risk of side effects, including irritant dermatitis and exogenous ochronosis. Meanwhile, kojic acid is known to cause dermatitis, sensitization, and erythema [6]. Thus, new alternatives with minimal side effects are needed to treat hyperpigmentation. Red seaweed is one of the potential sources of ingredients as an antioxidant and inhibitor of tyrosinase activity. This study aims to test the antioxidant activity using the DPPH, FRAP, and TBA-MDA methods, as well as the tyrosinase enzyme inhibition activity of *K. alvarezii* extracted with 70% ethanol, ethyl acetate, and n-hexane solvents.

2 Methodology

2.1 Preparation of Sample and Extraction

Seaweed was obtained from the Lombok cultivation center, Nusa Tenggara Barat, which is geographically located at 8°25'41.8" LS–8°45'00.7" N and 115°47'59.1"–116°45'41.4" E. The seaweed was cleaned and dried to make a 60-mesh sample powder and the water content was measured. 70% ethanol, ethyl acetate, and n-hexane were used as solvents for extraction by the maceration method (1:10 w/v) for 3x24 hours. The extract was concentrated with a rotary evaporator and the extract yield was calculated.

2.2 Determination of Total Phenolics and Flavonoids Content

Total phenolic analysis used the Folin-Ciocalteu method, with gallic acid standards (10, 25, 50, 75, 100, and 125 ppm). Samples were read for their absorbance at a maximum wavelength of 748 nm. Total phenolics were expressed in milligrams of gallic acid equivalents per gram of extract (mg GAE/g CE). Flavonoid testing used the $AlCl_3$ method with quercetin standards (120, 240, 360 480, and 600 ppm). The test was repeated three times. Samples were read for their absorbance at 423 nm using nano spectrophotometer (SPECTROstar Nano-BMG Lab Tech). Results were expressed in milligram of quercetin equivalent per gram of extract (mg QE/g CE).

2.3 Determination of Antioxidant Capacity

Antioxidant capacity was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing/antioxidant power (FRAP) methods. DPPH compounds are stable free radicals that show maximum absorption at 515 nm. A total of 100 µL of the extracted sample was mixed with 100 µL of DPPH 125 µM. The solution was incubated, and then the absorbance of the

solution was measured. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxyl acid) was used as a standard (10, 20, 30, 40, and 50 µM). For the FRAP test, 300 µL of FRAP solution was added to 10 µL of the sample. The mixture was incubated, and then the absorbance of the solution was measured at a wavelength of 593 nm. Trolox standards (100, 200, 300, 400, and 500 µM) were used in the FRAP test. The test was repeated three times. Absorbances were read using nano spectrophotometer (SPECTROstar Nano-BMG Lab Tech). The results of antioxidant activity were expressed in micromoles of trolox equivalents per gram of extract (µmol TE/g CE).

2.4 Determination of Lipid Peroxidation Inhibition Activity

Lipid peroxidation inhibition using the TBA-MDA method. Malondialdehyde (MDA) was measured by mixing the extract with linoleic acid (Sigma Aldrich) and then incubated for 10 days. Then, 20% trichloracetic acid (TCA) (Sigma Aldrich) and 1% thiobarbituric acid (TBA) (Sigma Aldrich) were added. The mixture was heated in a water bath at 100^{o}C for 15 minutes, cooled, and then centrifuged. After that, the supernatant was read for absorbance at a wavelength of 532 nm using nano spectrophotometer (SPECTROstar Nano-BMG Lab Tech). α-tocopherol 0,2 mg/mL was used as a standard.

2.5 Determination of Tyrosinase Inhibition Activity

Tyrosinase inhibition was determined on the reaction mixture consisted of 50 mM sodium phosphate buffer (pH 6,5), 333 U/mL fungal tyrosinase (Sigma Aldrich), and 2 mM L-tyrosine (Wako Pure Chemical Corporation, Japan). The mixture was incubated at room temperature for 30 minutes. Absorbance was measured at a wavelength of 492 nm using ELISA reader (Epoch BioTek). The extract was tested at 10 mg/mL in three replicates. Kojic acid 0,2 mg/mL was used as a positive control.

2.6 Statistical Analysis

Statistical analysis was performed using SPSS software. One-way analysis of variance (ANOVA) was used, followed by Tukey's test. Significant differences were considered at P values less than 0,05.

3 Results and Discussion

3.1 Yield and Phytochemical Analysis

The powder of *K. alvarezii* obtained through sieving was then tested for water content. The water content of the sample powder was 9,367±0,568%. Referring to **Table 1**, 70% ethanol produced a higher extract yield (21,329±1,061%) than ethyl acetate and n-hexane solvents. The ANOVA results on total phenolics and flavonoids showed a significant difference in results ($p<0,05$). The highest total phenolics were found in the ethyl acetate extract with an amount of 19,985±0.699 mg GAE / g CE, while the highest total flavonoids of 4,570±0,091 mg QE/g CE were obtained from the 70% ethanol extract.

Table 1. Yield and phytochemical test of *K. alvarezii* extract

Solvent	Yield (%)	Total phenolic content (mg GAE/g CE)	Total flavonoid content (mg QE/g CE)
70% Ethanol	21,329±1,061	3,500±0,095[b]	4,570±0,091[c]
Ethyl acetate	0,949±0,642	19,985±0,699[c]	3,027±0,114[b]
n-hexane	1,47±0,217	1,159±0,071[a]	0,778±0,023[a]

Numbers in the same column followed by the same letter indicate no significant difference based on Tukey's test (α=0,05).

The yield of 70% ethanol extract in this study was higher than the experiment conducted by Bhuyar *et al.* [7], which obtained a yield of 15,469% with 70% ethanol extract. Meanwhile, Hamid *et al.* [3] through their research results, obtained a yield of ethanol extract of 2,95±0,04%, which was also lower than this study. The high yield of 70% ethanol extract indicates the large number of metabolite compounds extracted compared to other solvents. The ethanol extract of *K. alvarezii* contains alkaloids, glycosides, phenolics, flavonoids, tannins, carbohydrates, proteins, and amino acids [8]. The yield of ethyl acetate and n-hexane in the study by Hamid *et al.* [3] was obtained as much as 2,98±0,01% and 0,68±0,02%. The solvent's polarity significantly affects the extract's yield. Compounds such as polysaccharides, proteins, and peptides contribute to the high yield of polar solvent extraction.

Ethyl acetate is a semipolar solvent that can attract polar and nonpolar phenolic compounds, resulting in high total phenolics. Generally, phenolic forms containing hydroxyl groups are soluble in polar solvents, while phenolics with many methyl groups are soluble in nonpolar solvents [9]. However, the total flavonoid results indicate that the flavonoid group does not dominate the phenolic group extracted with ethyl acetate.

The total flavonoid results show that flavonoid compounds increase with increasing solvent polarity. According to Chaves *et al.* [10], a mixture of water and organic solvents, such as 70% ethanol, can increase the total flavonoids that are polar. Total flavonoids increase with increasing solvent polarity. However, there is a significant decrease at very high polarity levels. This shows that although polar solvents can effectively extract metabolite compounds, solvents that are too polar may not be optimal for all phytochemicals.

3.2 Antioxidant Capacity

The highest DPPH antioxidant capacity was obtained in 70% ethanol extract (9,785±0,084 µmol TE/g CE), followed by ethyl acetate extract (9,540±0,175 µmol TE/g CE), and n-hexane extract (1,298±0,033 µmol TE/g CE) (**Fig. 1**). Tukey's test showed no significant difference between the antioxidant capacity of 70% ethanol extract and ethyl acetate extract (α=0,05). In the FRAP antioxidant capacity results, ethyl acetate extract had the highest antioxidant activity of 36,550±0,127 µmol TE/g CE, followed by 70% ethanol extract of 20,661±0,173 µmol TE/g CE, and n-hexane extract of 2,883±0,210 µmol TE/g CE.

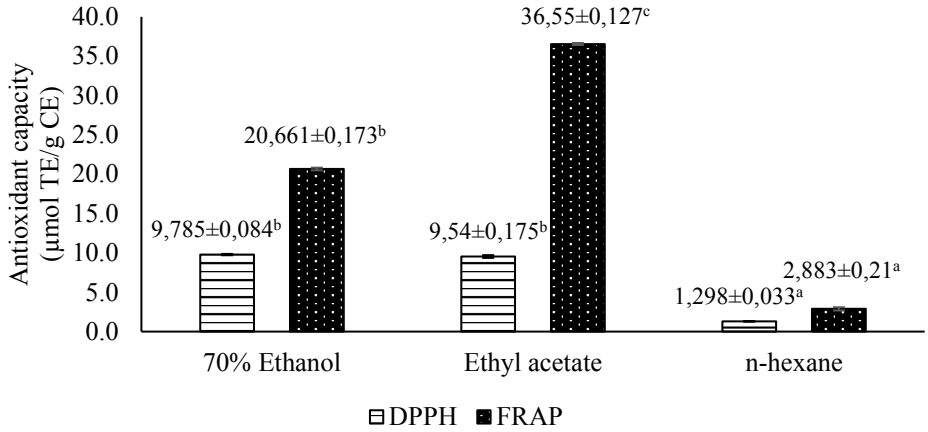

Fig 1. DPPH and FRAP antioxidant activities of *K. alvarezii* extract. Numbers in the same test followed by the same letter indicate no significant difference based on Tukey's test (α=0,05).

The ability of antioxidant compounds to reduce DPPH radicals occurs through a hydrogen transfer mechanism. Single electrons from nitrogen atoms in DPPH are reduced to hydrazine (DPPH-H) by taking hydrogen atoms from antioxidants. Meanwhile, the FRAP test method examines how antioxidant compounds reduce Fe^{3+} to Fe^{2+} through an electron donor mechanism. These results show that 70% ethanol and ethyl acetate extracts have the same activity in hydrogen transfer to reduce DPPH free radicals. 70% ethanol extract can contain antioxidants such as flavonoid glycosides, which act as strong hydrogen donors [11].

The FRAP test results align with the high phenolic content obtained in ethyl acetate extract. Phenolics are excellent antioxidants, which play an important role in reducing oxidative stress by reducing free radicals and preventing cellular damage. Ethyl acetate with semipolar properties attracts other compounds, such as non-flavonoid phenolics, terpenes, and tannins, which are effective as electron donors [2].

The low antioxidant capacity of n-hexane extract is because nonpolar solvents only extract a small amount of phenolic and flavonoid compounds, which are important antioxidants. In addition, other things can also be caused by the fact that the free radicals used are not soluble in nonpolar solvents [11]. The results of the antioxidant capacity against FRAP are generally higher than DPPH show that the antioxidant compounds extracted from *K. alvarezii* play a greater role in the donor transfer mechanism.

3.3 Lipid Peroxidation Inhibition Activity

The extract with a concentration of 10 mg/mL showed inhibitory activity in the 84–88% range, with the highest inhibition in the 70% ethanol extract (88,663±0,246%) (**Fig. 2**). Based on the results of the Tukey test, the 70% ethanol extract was not significantly different from the positive control of 0,2 mg/mL α-tocopherol (90,263±1,073%) (α=0,05). This indicates that the 70% ethanol extract of *K. alvarezii* has the potential to be used to inhibit MDA formation. Meanwhile, the ethyl acetate and n-hexane extracts also did not show significant differences (α=0,05).

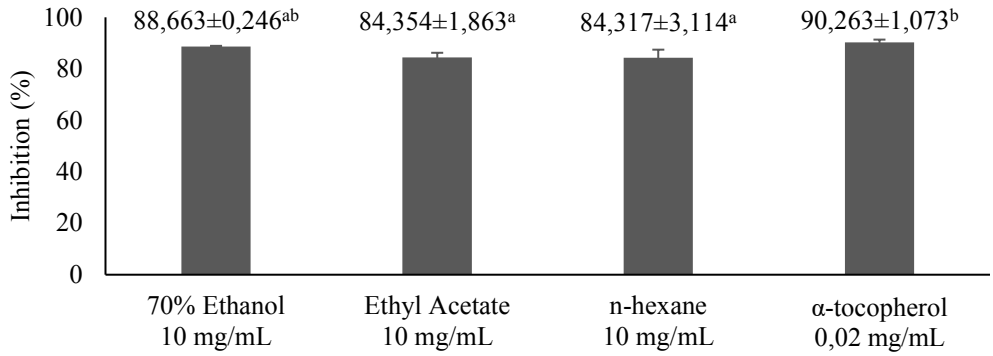

Fig 2. Lipid peroxidation inhibition activity of TBA-MDA method of *K. alvarezii* extract. Numbers followed by the same letter indicate no significant difference based on Tukey's test (α=0,05).

These results align with in vivo studies in mice, showing that ethanol extract of *K. alvarezii* can reduce MDA levels in the liver of lead-contaminated mice. Lipid peroxidation inhibition assay with linoleic acid as the oxidation target resulted in relatively good nonpolar antioxidant activity. Lipophilic nonpolar antioxidant compounds such as carotenoids and several polyphenols can be extracted in less polar solvents, which show potent antioxidants. *K. alvarezii* contains photosynthetic pigments such as chlorophyll a, chlorophyll d, β-carotene, and xanthophyll. Pérez-Gálvez *et al.* [12] stated that carotenoids as lipophilic antioxidants can react through different mechanisms, such as electron transfer, hydrogen reduction, and carotenoid-radical formation, depending on the structural composition of the carotenoid.

3.4 Tyrosinase Inhibition Activity

The extract with a 10 mg/mL concentration showed low inhibitory activity compared to kojic acid 0.01 mg/mL. The results of tyrosinase inhibition by kojic acid were 56,833±1,121%, while inhibition by 70% ethanol extract, ethyl acetate extract, and n-hexane extract were 43,016±3,064%, 41,474±1,365%, and 37,515±0,467% respectively as shown in **Fig. 3**.

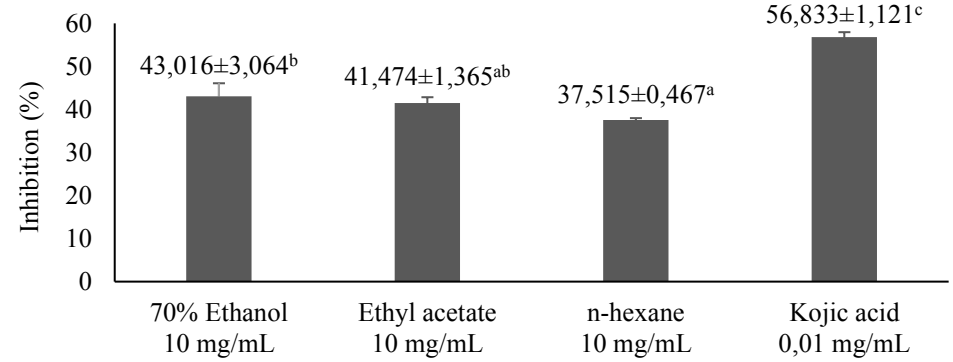

Fig 3. Tyrosinase inhibition activity of *K. alvarezii* extract. Numbers followed by the same letter indicate no significant difference based on Tukey's test (α=0.05).

The inhibition of 70% ethanol extract in this study was higher than the results obtained by Dolorosa *et al.* [5] on 15% *K. alvarezii* extract in the form of a cream preparation, with tyrosinase inhibition of 13,05 ± 1,97%. The inhibitory activity of 70% ethanol extract of *K. alvarezii* was lower when compared to other types of seaweed such as *Ulva intestinalis* (96,04%), *Sargassum polycistum* (94%), and *Lobohora challengeriae* (94%), but better than

Caulerpa racemosa extract (<30%) and *Caulerpa lentillifera* (<15%) [13]. This shows that *K. alvarezii* can be an option for use in tyrosinase inhibition.

Kojic acid, a pyranone compound with a hydroxyl group, is used as a standard because it shows great tyrosinase inhibitory activity. Kojic acid can bind copper at the enzyme's active site, showing a competitive inhibitory effect. Phenolics and polyphenols are known to have inhibitory abilities through the same mechanism as kojic acid. This group of compounds can interact with copper ions at the active site and are structurally similar to tyrosinase substrates [14]. This supports tyrosinase inhibitory activity in 70% ethanol and ethyl acetate extracts.

On the other hand, hydrophobic steroids and long-chain fats have the potential to inhibit tyrosinase. The trilinolein compound isolated from sake dregs is known to have weak tyrosinase inhibitory activity. The inhibition mechanism on steroids and fats is thought to occur non-competitively by binding the compound to several tyrosinase sites [15]. Thus, this can support the tyrosinase inhibition activity in n-hexane extract, which is not significantly different from that of ethyl acetate extract.

4 Conclusions

K. alvarezii extract showed varying antioxidant and tyrosinase inhibition activities. In general, *K. alvarezii* extract is active as an antioxidant through an electron transfer mechanism. This is shown in the FRAP antioxidant test method results, which are higher than the DPPH method. The results of the DPPH antioxidant capacity in 70% ethanol and ethyl acetate solvents did not show any significant differences. Based on the results of the FRAP test, ethyl acetate has the highest antioxidant capacity. Ethanol 70% obtained the best results in lipid peroxidation and tyrosinase inhibition activity. Meanwhile, the n-hexane extract showed the lowest activity in each test. Thus, compounds from 70% ethanol and ethyl acetate extracts of *K. alvarezii* can be used as antioxidants and tyrosinase inhibitors.

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