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Research Article
Phytochemical and Antioxidant Activities of *Eucheuma spinosum* as Natural Functional Food from North Sulawesi Waters, Indonesia

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Abstract

Background and Objective: Research to find functional food from new natural sources has caught the attention of many researchers through the characterization of phytochemicals and biological activities. One potential source of natural ingredients is the red alga *Eucheuma spinosum*, which has been used as a daily source of natural food. The purpose of this study was to obtain a prospective new source of natural antioxidants from various extracts of tropical red alga (*E. spinosum*) through several tests, which were used as determinants of whether the alga can be used as a functional food source. **Materials and Methods:** Algal sample was extracted with organic solvents (methanol, n-hexane, ethyl-acetate and water) and purified by a combination of normal and reverse phase chromatography methods. **Results:** The algal extracts had antioxidant compounds based on free radical scavenging activities using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and superoxide dismutase (SOD). The ethyl acetate extract of *E. spinosum* scavenged DPPH and SOD free radicals, so that this extract was indicated contained powerful antioxidants. The result of the isolation of the antioxidant compound showed the presence of pure compound 3-(3-methoxyphenyl) propanal. **Conclusion:** This study concluded that red algae *E. spinosum* contained natural antioxidants which have the potential to be developed as a functional food and disease prevention and treatment. In addition, the components of these antioxidant compounds from the algae have the potential to be used as natural sources of new functional ingredients.

Key words: Functional food, red alga, *Eucheuma spinosum*, antioxidant, free radicals, sea weed, seaweeds

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In the last few decades, research on functional food and its bioactive compounds has been done extensively because it provides health benefits through the content of bioactive compounds[1]. These compounds have benefits in the prevention and treatment of degenerative diseases and metabolic disorders. The content of bioactive ingredients in food, although in small amounts, contributes to regulating biological mechanisms. This fact shows the potential use of plant bioactive compounds, including algae, as new sources of functional food ingredients and food preservatives.

Eucheuma spinosum is the most important source of many biologically active metabolites, compared to other algae[2]. This alga contains bioactive compounds, for example, flavonoids, alkaloids, saponins, tannins and their derivatives which have antibacterial properties[3] and antioxidants[4]. Antioxidants are usually added to food to slow down oxidative decline and prevent chronic diseases in the body[5]. This research was conducted to find a new source of natural antioxidants from tropical alga *E. spinosum* using 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and superoxide dismutase (SOD). Phytochemical screening was conducted to determine the presence of secondary metabolites of the alga.

MATERIALS AND METHODS

Specimen collection and determination: Red alga *E. spinosum* was cultivated and collected in April, 2018 from Arakan waters, North Sulawesi, Indonesia. The specimen was identified at the Plant Taxonomy Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University, Bandung, Indonesia. This research project was conducted from April-August, 2018.

Extraction method: The fresh alga was well washed in freshwater on the day it was collected and chopped into smaller pieces. The sample was extracted with methanol (analytical grade-Merck Millipore-Germany) and then partitioned in succession with water-n-hexane (analytical grade-Merck Millipore-Germany) and water-ethyl acetate (analytical grade-Merck Millipore-Germany). Then, the supernatant of the mixture was filtered through a Whatman filter (Whatman Clifton, NJ, USA), concentrated in a rotary vacuum evaporator (Buchi, Zurich, Switzerland) and made in a series of concentrations for phytochemical and antioxidant screening with DPPH and SOD activity tests.

Preliminary phytochemical screening: Phytochemical screening was conducted to determine secondary metabolite compounds including alkaloids, terpenoids and flavonoids. The extraction was performed using methanol, n-hexane, ethyl acetate (EtOAc) and water (H_2O) solvents by following methods of Harbone[6].

DPPH activity assay: The percentage of antioxidant activity (%) of *E. spinosum* extract was assessed by DPPH free radical test. Measurement of DPPH radical binding activity was carried out according to the method described by Williams *et al.*[7]. The sample was reacted with stable DPPH radicals in an ethanol solution. The reaction mixture consisted of 0.5 mL of sample, 3 mL of absolute ethanol and 0.3 mL of DPPH radical solution (0.5 µM in ethanol). DPPH will bind the hydrogen donated by antioxidants contained in the sample. The binding reaction was characterized by a change in color from dark purple to light yellow. The change in color was observed at absorbance with a wavelength of 517 nm after 100 min of reaction using a UV-Vis Spectrophotometer (Shimadzu UV-Vis 1800-Japan). A mixture of ethanol (3.3 mL) and a sample (0.5 mL) are presented as negative controls. A positive control solution was prepared by mixing ethanol (3.5 mL) and a radical DPPH solution (0.3 mL). Quercetin and catechin were used as positive controls. The percentage of scavenging activity (%) was determined based on Mensor *et al.*[8] as follows:

$$\text{AA (\%)} = 100 - \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{negative control}}}{\text{Abs}_{\text{negative control}}} \times 100$$

Superoxide dismutase (SOD) activity assay: The SOD activity of the sample was evaluated using the indirect method of riboflavin photoreduction. This method involves a competitive reaction between complex and reduced NBT (nitroblue tetrazolium) for O_2% produced by riboflavin in lighting at room temperature (25°C). The sample mixture (240 µL) contained eleven different sample concentrations, 6 µM riboflavin, 0.8 µM N,N,N',N'-tetramethylethylenediamine (TMEDA) in 0.016 M phosphate buffer (pH 7.4) and 85 µM NBT. The reaction was stopped by turning off the lights after 15 min (4 fluorescence tubes, Philips TLD/20 W, a distance of 20 cm) and NBT absorbance was measured at λ 560 nm with Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific GmbH, Germany)[9].

Structure determination of active pure compound: The pure compound was isolated from EtOAc extract and its structure was characterized by spectroscopic methods. The ^{1}H and ^{13}C NMR spectra were measured using JEOL JNM ECA 500 MHz NMR Nuclear magnetic resonance spectroscopy (Akishima, Tokyo Japan), which operates at 500 and 125 MHz, respectively.

RESULTS AND DISCUSSION

Rendement and phytochemical composition of *E. spinosum* extracts: As much as 4.1 kg fresh *E. spinosum* was extracted with methanol for 3×24 hrs and continued with partitioning using n-hexane-water and EtOAc-H_2O. The extract results for each solvent are as follows: MeOH (12.9 g), n-hexane (0.0649 g), EtOAc (0.4969 g) and H_2O (8.5265 g). It was observed that the MeOH solvent produced the highest yield of 12.9 g. The extraction results using EtOAc solvent also produced a higher yield than using n-hexane solvent. This means that polar solvents attracted more compounds than non-polar solvent. The composition of secondary metabolites according to the phytochemical screening results is presented in Table 1.

Phytochemical screening results showed that tannin was found in all *E. spinosum* extracts. Methanol extract also contained steroids and triterpenoids, n-hexane extract contained alkaloids and EtOAc extract contained flavonoids, steroids, triterpenoids and saponins. These results provide strong evidence that alga *E. spinosum* is a potential source of natural bioactive compounds. This result is supported by previous research which showed that *E. spinosum* contained important antioxidant compounds[10].

Scavenging activities of the extracts: The present study confirmed that n-hexane, EtOAc and H_2O extracts had antioxidant activity using DPPH and SOD. Test results showed that algal extract scavenged DPPH free radicals with increased concentration, especially in EtOAc extract. However, water, EtOAc and n-hexane extracts were active with inhibition activity (IC_{50}) of 2,478.1, 402.8 and 1,537.1 ppm, respectively, as shown in Table 2.

The EtOAc extract showed the highest DPPH radical scavenging activity with IC_{50} of 402.8 ppm compared to reference compounds quercetin and catechin with IC_{50} of 20.98 and 91.82 ppm, respectively. The activity of these reference compounds is less effective than the activity of EtOAc extract[11,12]. In a previous publication, it was reported that methanol extract from *E. spinosum* and *E. cottonii* scavenged DPPH free radicals with IC_{50} of 75.27 and 64.73 ppm, respectively[13]. According to the results of the phytochemical analysis, secondary metabolites such as flavonoids, steroids, terpenoids, saponins and tannins contained in EtOAc extract had linear correlations with their antioxidant activities.

Increased concentrations in *E. spinosum* extract scavenged SOD free radicals, especially in EtOAc extract. An increase in the antioxidant activity of algae extracts for H_2O, EtOAc and n-hexane depended on each concentration. The IC_{50} of each algal extract was 155, 25 and 870 ppm, respectively, as shown in Table 3.

Analysis of antioxidant based on SOD free radical scavenging activity showed that EtOAc extract was also the most active compared to other extracts, with IC_{50} of 25 ppm, while SOD activity for quercetin and catechin reference compounds were 3.33 and 26.51 ppm, respectively. A study

Table 1: Phytochemical composition the *E. spinosum* extracts

		Samples			
Secondary metabolites	Reagent	MeOH	n-Hexane	EtOAc	H_2O
Flavonoid	HCl+Mg	-	-	-	-
	H_2SO_4 (2N)	-	-	-	-
	NaOH (10%)	-	-	+	-
Alkaloid	Pereaksi Dragendorff	-	+	-	-
Steroid	Lieberman-	+	-	+	-
Triterpenoid	Burchard	+	-	+	-
Saponin	HCl+H_2O	-	-	+	-
Tanin	$FeCl_3$ (1%)	+	+	+	+

Table 2: Antioxidant DPPH free radical scavenging activities of algal extracts

	Inhibition activity (IC_{50}/ppm)			
Samples	n-Hexane	EtOAc	H_2O	MeOH
Extracts	1,537.1	402.80	2,478.1	-
3-(3-methoxyphenyl)propanal	-	87.97	-	-
Quercetin	-	-	-	20.98
Catechin	-	91.82	-	-

Table 3: Antioxidant SOD radical scavenging activities of algal extracts

	Inhibition activity (IC_{50}/ppm)			
Samples	n-Hexane	EtOAc	H_2O	MeOH
Eucheuma spinosum extract	870	25	155	160
Quercetin	-	-	-	5
Catechin	-	-	-	13

reported that *E. cottonni* methanol extract scavenged SOD free radicals with IC_{50} of 42.52 ppm[14]. This shows that the *E. spinosum* EtOAc extract was more active than the methanol extract of *E. cottonni*. Other data showed that extracts ethyl acetate of *Pinus maririma*, commercial pine, *Quercus robur*, *Cinnamomum zeylanicum* and *Ilex paraguariensis* scavenged DPPH free radical, successively at 94.51, 92.79, 88.60, 84.43 and 71.75%. Ethyl acetate extract also scavenged SOD free radicals with a value of 60.32, 53.48, 81.20, 51.79 and 52.44%, respectively[15]. The data indicate that EtOAc extract was more active in scavenging free radical DPPH and SOD.

Ethyl acetate extract could extract more phenols as evidenced by the presence of tannins, flavonoids, triterpenoids and steroids. The relatively high molecular weight tannin group exhibits greater antioxidant activity than simple phenols[16]. The hydroxyl phenol group was reported to have a major role in antioxidant activity, especially flavonoids[17-19]. The combination of phenolic compounds and saponins (crude extracts) had higher antioxidant activity than saponins isolated from the same source. This shows that other molecules increased the antioxidant activity of the extract[20]. Steroid compounds showed the highest flushing activity in the generation of intracellular ROS[21]. The triterpenoid content detected in Merlot and Syrah had antioxidant activity[22].

DPPH is a stable free radical that is widely used to evaluate natural antioxidants in algae and algal products, because of its stability, simplicity and reproducibility. Some antioxidants can react slowly and some even do not react with DPPH[23]. DPPH as free radical by the delocalization of the spare electron contributes to changing the deep purple color to pale yellow after a reduction in the substrate[24]. SOD catalyzes the conversion of Super Oxide (SO) to hydrogen peroxide and oxygen, an important reaction that removes SO in cells. Super Oxide is very reactive and can cause cell damage and excessive amounts trigger reactions that cause damage to biologically important macromolecules, such as DNA, lipids and proteins[25,26].

Antioxidants by their mechanism of action are grouped into primary and secondary antioxidants. Primary antioxidants are the main antioxidants that give hydrogen atoms (H) quickly to radical compounds. Radical compounds formed produce lipid derivatives and antioxidant radicals (A*). It acts as a hydrogen atom donor to fat-free radicals, to reshape molecules. So, if antioxidants are given to prevent the formation of new radicals, they will inhibit the process of auto-oxidation[27].

Isolation and structure determination of antioxidant compound-1 from the EtOAc extract of *E. spinosum*: The active antioxidant extract from the EtOAc fraction was selected for further separation of the active compound. Ethyl acetate extract (1.1 g) was chromatographed on Silica G 60 and eluted using a stepwise 5% gradient of n-hexane-EtOAc to produce two active fractions of I or compound-1 (165 mg) and II (178.8 mg).

The pure compound-1 was isolated as a yellowish-brown solid that was soluble in methanol. The ^{13}C-NMR spectrum showed 10 carbon signals including 1 (one) carbonyl carbon at d_C 192.7 ppm and 6 (six) olefinic carbon 156.7, 133.5, 133.2, 130.3, 117.1 and 116.2 ppm. Another signal was identified for two methylene carbon at δC 30.1 and 46.3, together with 1 (one) methoxy carbon at δC 56.4 ppm. Correlated with carbon data, it was found from the ^{1}H-NMR spectrum that showed 4 (four) aromatics and 1 (one) proton aldehyde (δH 9.69), respectively. From the J value of 8.4 ppm, two protons at δH 7.76 (H-5') and 7.70 (H-6') were determined at the ortho position, while the other two protons at δH 6.68 (H-4') and 6.90 (H-2') ppm are in the meta position (J = 9.1 ppm). From the HMBC spectrum, the methoxyl group was attached to C-3', with a peak correlation between the methoxyl proton and carbon C-3', while one of the two methylene groups H_2-2 and H_2-3 was constantly attached to the carbon side of the olefin C-1', the other side was attached to olefinic carbon C-1′ from the aldehyde functional group. Based on the analysis of 1D and 2D-NMR data, the structure of compound-1 was suggested as 3-(3-methoxyphenyl) propanal (synonym: 3-(3-methoxy phenyl) propionaldehyde benzene

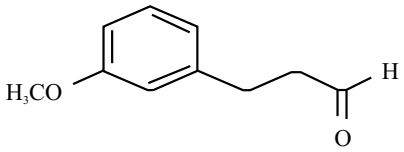

Fig. 1: Structure of compound 1 [3-(3-methoxyphenyl) propanal

propanal, 3-methoxy benzene propanal), as seen in Fig. 1. Confirmation of structure 1 as reported in the publication corresponds to a synthetic compound of the same compound from 3-methoxycinnamic[28]. Based on references from NMR data, pure-active compounds isolated from tropical alga *E. spinosum* is reported for the first time in this paper with the following chemical shift values: δ_C 192.8 (C1), 46.3 (C2), 30.1 (C3), 133.2 (C1'), 117.1 (C2'), 156.7 (C3'), 116.2 (C4'), 133.5 (C5'), 130.3 (C6'), 56.4 (OMe-3'); δ_H 9.69 (1H, s, H1), 2.74 (2H, t, J = 3.25 and 1.3, H2), 2.10 (2H, s, H3), 6.90 (1H, d, 9.1, H2'), 6.68 (1H, d, J = 8.45, H4'), 7.76 (1H, d, 8.4, H5'), 7.00 (1H, d, 8.4, H6'), 3.90 (2H, s, -OMe).

Antioxidant activity of 3-(3-methoxyphenyl) propanal (compound-1) was tested against DPPH. Data show that compound-1 scavenged DPPH free radicals with IC_{50} of 87.97 ppm. The antioxidant activity of pure compound-1 was observed at 87.97 ppm. When compared with IC_{50} of the reference compounds, this pure compound was active like BHT with IC_{50} of 84.15±3.82 μg mL^{-1} [29], but less active than vitamin C with IC_{50} of 21 μg mL^{-1} [30].

Various food antioxidants have been classified into different categories based on their chemical structure and function, which are water-soluble including citrate, norbixin, betalains, mostly phenolics, flavonoids and anthocyanins and fat-soluble components such as carotenoids, tocopherols, terpenoids and vitamin E[31]. Foods that are rich in bioactive compounds play important roles in the prevention and treatment of chronic gastrointestinal (GI) diseases[32]. Functional foods have complex matrices and their composition of bioactive compounds requires careful assessment because potential risks can arise from the isolated material. It is known that plants produce and accumulate a variety of typical chemical compounds, which are usually in low concentrations[33].

The alga *E. spinosum* which is rich in bioactive compounds can be used as a functional food. This alga contained 3- (3-methoxyphenyl) propanal which according to Alghamdi *et al.*[34], this compound were detected in soybean and had antibacterial activity. Natural antioxidants from marine functional food products are reported to be an alternative way to prevent or treat metabolic diseases, such as diabetes, Alzheimer's and stroke[35].

In many parts of Indonesia, the alga *E. spinosum* is used as food and eaten raw or cooked. It's application as a functional food ingredient containing natural antioxidants, among others, by adding its extract to processed food products, or as a raw material for medicine or for the prevention of certain diseases. This study is a preliminary evaluation aimed at determining the antioxidant activity of *E. spinosum*. Henceforth, it is necessary to study the content of other antioxidants that have the potential to act as antidotes to free radicals, as well as the process of making food products with the addition of natural antioxidants.

CONCLUSION

In this study, red alga *E. spinosum* was identified as a potential source of natural antioxidants that actively scavenged free radicals DPPH and SOD, so that it can be used as an alternative antioxidant as a functional food for disease prevention and treatment. The pure antioxidant compound found in *E. spinosum* extract was predicted as 3-(3-methoxyphenyl) propanal based on its chemical composition.

SIGNIFICANCE STATEMENT

This study discovers that the compound 3-(3-methoxyphenyl) propanal found in *E. spinosum* extract is a pure antioxidant compound which is thought to have a significant contribution to the high antioxidant activity of this algal extract. This study will help researcher to uncover more potential of this alga as a functional food. This study will help researchers to uncover other types of pure antioxidants contained in this alga that may have a high ability to ward off free radicals. In addition, it can also be used as a reference for formulating this alga as other food additives.

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