Effect of extraction solvent on total phenolic content, total flavonoid content, and antioxidant activity of Bulung Sangu (Gracilaria sp.) Seaweed

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Abstract. Bulung sangu (Gracilaria sp.) is commonly consumed as vegetable in Bali. Bulung sangu as other red macroalgae (Rhodophyta) is a source of beneficial nutrient for health. In this study, water and various concentrations (50%, 75% and 100%) of methanol, ethanol, and acetone in water were used as solvent in extraction of bulung sangu. The antioxidant activity, total phenolic content, and total flavonoid content of crude extract of bulung sangu were investigated using various in vitro assay. The extract obtained by 75% of aqueous methanol produced higher extraction yield (27.390 ± 0.414 %). Highest total phenolic content was obtained by the using 100% of acetone (36.738 ± 1.062 mg galic acid equivalent/g ). The extract obtained by 100% of ethanol showed highest total flavonoid content (45.933 ± 0.563 mg quercetin equivalent/g). The same extract also exhibited the strongest antioxidant activity indicated by lowest half maximal inhibitory concentration (IC50) (13.603 ± 0.413 µg/ml) evaluated by using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity. This IC50 was lower than IC50 of ascorbic acid (18.593 ± 0.135 µg/ml). These results produce the suitable solvent in obtaining optimum phenolic and flavonoid content of bulung sangu. Likewise, the antioxidant activity results indicate that bulung sangu is useful in dietary application with a potential to reduce oxidative stress.

1. Introduction

Nowadays, the consumption of herbal medication and natural products has been become a developing trend. Many plants, especially medicinal plants, have been studied for their antioxidant activity. In Indonesia, many medicinal plants that were previously used empirically, have now scientifically proven for their biological activity. Indonesia as a high biodiversity country has a great opportunity to provide various medicinal plants to be explored, especially the potential plants with high antioxidant activity.

The intake of antioxidant supplements is associated with a reduced risk of degenerative diseases such as cardiovascular disease and cancer[1]. Poor antioxidant intake was also reported as a factor associated with incidence of frailty and an increased risk of clinically significant depressive symptoms[2,3]. Oxidative stress contributes to the manifestation of various health complications[4]. Antioxidant...
compounds have been used to prevent or as an adjunct therapy in various diseases[5]. Antioxidant-containing compounds are reported play an import role in providing a positive response to the body against various conditions such as inflammation, hypercholesterolemia, and cardiovascular disorders [6–8].

There are three main classes of phytochemical components: terpenoids, phenolics and alkaloids[9]. Phenolic compounds are considered to be the most important metabolites for food applications among the three groups. Phenolic compounds are also the most studied compounds[10]. Plant polyphenols are secondary metabolites in the form of one or more hydroxyl groups attached to an aromatic ring. More than a thousand types of polyphenol molecules have been identified in plants, including the inconsumable types. Polyphenol plants consist of two main groups: flavonoids and non-flavonoids. Several types of flavonoid compounds are stilbene, phenolic acid, saponins and tannins[11]. Polyphenol group compounds show strong activity to against various diseases associated with oxidative stress[12,13].

Several techniques can be used to isolate antioxidant compounds in plants such as soxhlet extraction, maceration, supercritical fluid extraction, subcritical water extraction, and ultrasound-assisted extraction[14]. The success of the isolation process and the amount of extract produced depends on method used and extraction solvent. Antioxidant compounds find in various forms with different levels of polarity[15]. Combinations of water and organic solvents such as ethanol, methanol and acetone are considered the most suitable solvent to isolate polyphenol. Ethanol is known to be able to extract polyphenol compounds, and relatively safe for consumption. Methanol is generally more efficient for extracting polyphenols with low molecular weight, while acetone is effective for extracting polyphenols with high molecular weight[16]. Do et al. found that aromatic Limnophila extracted with 100% ethanol produced the highest antioxidant activity, phenolic and flavonoid levels compared to methanol and acetone. While extraction with 100% acetone produced the highest extraction yield[15]. Likewise, Wang et al. reported the more effective use of water ethanol in flavonoids extraction, as compared to water methanol and aqueous acetone[17].

Bulung sangu is a red macroalgae from the genus Gracilaria. Bulung sangu is widespread in Bali, and usually consumed as a substitute for vegetables. The ethanol extract of bulung sangu formulated in cream preparations shows high antioxidant activity with lower IC₅₀ as compared to ascorbic acid[18]. The antioxidant properties of the ethanolic extract of bulung sangu are also predicted to be related to the anti-inflammatory activity against UV-B radiation[6]. However, there have been no reports regarding the phenolic content and contribution of phenolic compounds to the antioxidant activity of bulung sangu. The purpose of this study was to determine the effect of solvents on the extraction of polyphenols from bulung sangu extract and to determine the antioxidant activity of the extract using the DPPH method.

2. Materials and methods

2.1. Macroalgae material and preparation

Bulung sangu (Gracilaria sp.) macroalgae used in this study was collected at Serangan, Bali. Fresh and red bulung sangu was cleaned from impurities and washed under running water. Clean bulung sangu was the chopped to reduce the size. The chopped sample was then oven-dried at a temperature of 40 for 3-7 days until a constant weight was obtained. Dried sample was blended and sieved in powder.

2.2. Extraction

Water, methanol, ethanol, and acetone were used in this research. Methanol, ethanol and acetone were used in various concentrations (50%, 75%, and 100%) resulting in ten different extraction solvent. 10 g of sample powder was macerated by 20 mL of extraction solvent at room temperature for a period of three days with regular shaking. After filtration, organic solvent was evaporated under vacuum to furnish dry extract. The crude extract was then stored at -20°C.
2.3. **Determination of polyphenol content**

2.3.1. **Total phenolic content.** The total phenolic content (TPC) for each extract was determined using the Folin-Ciocalteu method described by Do et al. and McDonald et al. with modifications [15,19]. The extract was diluted in distilled water to obtain the concentrations of 50 µg/mL. The calibration curve was established using gallic acid (0-60 µg/mL). The diluted extract and gallic acid (1.6 mL) was added to 0.2 mL of Folin-Ciocalteu reagent and mixed thoroughly for 3 minutes. 0.2 mL of 10% w/v sodium carbonate was added and the mixture was allowed to stand for 30 minutes at room temperature. The absorbance was measured at 760 nm using UV-VIS spectrophotometer. TPC was expressed as milligram gallic acid equivalent per gram extract (mg GAE/g extract).

2.3.2. **Total flavonoid content.** Total flavonoid content (TFC) for each extract was determined using aluminum chloride colorimetric method described by Do et al. and Chang et al. with modifications [15,20]. The extract was diluted in methanol to obtain the concentration of 100 µg/mL. The calibration curve was established using quercetin in concentrations of 0-100 µg/mL in methanol. Both 2.0 mL of the diluted extract and quercetin was mixed with 0.1 mL of 10% (w/v) aluminum chloride solution and 0.1 mM potassium acetate solution. The mixture was allowed to stand for 30 minutes at room temperature. The absorbance was measured at 415 nm using UV-VIS spectrophotometer. TFC was expressed as milligram quercetin equivalent per gram extract (mg QCE/g).

2.4. **Antioxidant activity**

The antioxidant activity was measured using DPPH. The DPPH solution was prepared in methanol by dissolving 6mg DPPH in 50mL methanol. Extract in various concentration (60-220 µg/ml) was mixed with 2.5 mL of DPPH solution, then incubated for 30 minutes in the dark at room temperature. The absorbance was read with a UV-Vis spectrophotometer at a wavelength of 517 nm. The percentage inhibition of radicals was calculated using the following formula:

\[
\% \text{ inhibition} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

Control absorbance (Acontrol) is the absorbance of DPPH solution without extract. Sample absorbance (Asample) is the absorbance of sample in DPPH solutions. Ascorbic acid was used as the positive control. Linear regressions were obtained for ascorbic acid and each extract, and used to calculate IC50. The half-maximal inhibitory concentration (IC50) was reported as the amount of antioxidant required to decrease the initial DPPH concentration by 50%.

2.5. **Statistical Analysis**

All analyses were done at least in triplicate. The values were presented as average values along with standard deviations. Comparison among all data were analyzed by using One-Way ANOVA followed by Post Hoc Test Fisher's Least Significant Difference (LDS). Linear regression correlation test was conducted to analyze the correlation of each dependent variable to dielectric constant of each solvent system used. All statistical analyzes used 95% of confidence level.

3. **Results and Discussion**

Bulung sangu is member of Gracilariaceae family wildly grows in Bali. Bulung sangu was obtained in intertidal zone, attached to rocks, plants or other seaweed, and is commonly found buried in sand. Bulung sangu is pink to dark brownish red (mahogany color). Exposure to sunlight can fade the dark color of bulung sangu, making it transparent green or white. Bulung sangu thallus is a clump with an irregular branching type, and cylindrical with sharpened talus edges, with the length ranges from 7-18 cm, diameter ranges from 1-1.5 mm and 0.1-0.5 mm for the tapered ends of the thallus. Bulung sangu
lives by attaching itself to plants or other seaweed using holdfast that resembles a root thus wrapping the thallus to the attached object (Figure 1.).

Figure 1. Thallus of bulung sangu (A). Bulung sangu lives by attaching itself to plants or other seaweed using holdfast (arrow), which has a root-like structure in a true plant (B), thus wraps the thallus on other seaweed (C).

3.1. Effect of solvent system in extraction yield

Medicinal plants provide the phytochemicals that play an important role in the development of conventional medicines. Most of the phytochemicals used are phenolic and flavonoids compounds which are reported for having many effects on health and cancer prevention[21]. The use of natural sources in the development and formulation of skin and beauty products has become an alternative to the use of conventional medicines and synthetic products, which has led to increased research on medicinal plants[22]. Research on phytochemicals and medicinal plants begins with pre-extraction and extraction procedures which are important stages in the isolation of bioactive constituents from plant material[23].

In the scope of the study, extraction experiments are design to optimize the most suitable extraction solvent to obtain phenolic compound in the bulung sangu macroalae. Extraction is the main step for recovering and isolating phytochemicals from plant materials. Several points affect the extraction process including extraction method, sample particle size, extraction solvent and the presence of interfering substance[24]. The yield of extraction depends on the solvent polarity, pH, temperature extraction time, and composition of the sample. Solvent with varying polarity affect the extraction yield as well. In the circumstance of the same extraction time and temperature, solvent and composition of the sample are knows as the most important parameters affecting the extraction yield[15]. Beside of the proper selection of extraction method, the solvent is fundamental to obtain the maximal extraction yield with minimal functional properties changes[25].

In this work, bulung sangu extracts were obtained by using water and different concentrations of aqueous methanol, ethanol and acetone. Extraction yield range from 8.433% for 100% acetone extract up to 27.390% for 75% aqueous methanol extract (Table 1). The difference in the extract yield due to the ability of different solvent to solve the extractable components, resulting the varied chemical
compositions of plants[26]. This lowest result is similar to the results reported by Do et al. in which extraction with 100% of acetone resulting in the lowest extraction yield among other extraction solvents[15]. The yield of extraction by various solvents decreased in this following order: 75% aqueous methanol > 50% aqueous ethanol > 75% aqueous methanol > 75% aqueous acetone > 75% aqueous ethanol > 100% methanol > distilled water > 50% aqueous acetone > 100% ethanol > 100% acetone. Statistically there were significant different among all extraction yield except for the water, 100% methanol, 75% aqueous ethanol, and 50% aqueous ethanol. The significant highest extraction yield produced by the extraction using 75% of aqueous methanol. Linear correlation showed the significant positive correlation of dielectric constant and extraction yield with correlation coefficient of 0.418. The results indicating that solvent polarity simultaneously and significantly correlated to extraction yield. The extraction efficiency favours the highly polar solvents. Presence of water in the solvent system increase dielectric constant increase as well as the extraction yield. This may be attributable to the higher solubility of protein and carbohydrates in water and methanol than either in ethanol and acetone[27]. Combination of water and organic solvent facilitate the isolation of both components soluble in water and organic solvent. Monteriro et al. reported the high biomass extracted by the usage of 50% aqueous methanol and ethanol[25]. Usage of methanol resulted in high extraction yield in several study[15,28,29].

| Solvent                  | Extraction Yield (%) | TPC (mg GAE/g) | TFC (mg QCE/g) | IC_{50}(µg/ml) |
|--------------------------|----------------------|----------------|----------------|---------------|
| Water                    | 20.893 ± 0.742±      | 4.723 ± 0.575± | 4.848 ± 0.411± | 622.305 ± 2.032± |
| 100% Methanol            | 21.138 ± 0.156±      | 25.045 ± 0.073± | 10.760 ± 0.463± | 38.030 ± 1.078± |
| 75% Aqueous Methanol     | 27.390 ± 0.414±      | 30.480 ± 0.304± | 17.458 ± 0.327± | 23.558 ± 1.242± |
| 50% Aqueous Methanol     | 24.465 ± 0.432±      | 15.362 ± 0.320± | 7.588 ± 0.206± | 81.273 ± 0.904± |
| 100% Ethanol             | 17.013 ± 0.250±      | 36.273 ± 0.241± | 45.933 ± 0.563± | 13.603 ± 0.413± |
| 75% Aqueous Ethanol      | 21.413 ± 0.648±      | 25.878 ± 0.125± | 15.155 ± 0.694± | 48.038 ± 1.969± |
| 50% Aqueous Ethanol      | 26.795 ± 0.821±      | 24.983 ± 1.123± | 13.223 ± 0.616± | 56.160 ± 1.582± |
| 100% Acetone             | 8.433 ± 0.611±       | 36.738 ± 1.062± | 25.415 ± 0.473± | 31.73 ± 0.743± |
| 75% Aqueous Acetone      | 22.325 ± 0.848±      | 34.950 ± 0.872± | 23.305 ± 1.087± | 22.093 ± 0.949± |
| 50% Aqueous Acetone      | 18.028 ± 0.621±      | 23.680 ± 1.100± | 13.835 ± 1.131± | 104.265 ± 1.210± |

Tabel 1. Extraction yield, TPC and TFC, and IC_{50} in DPPH radical scavenging activity of bulung sangu crude extract.

Different letters in superscript (±) indicating significant difference from one another (p < 0.05).
GAE = gallic acid equivalent; QCE = quercetin; TFC = total flavonoid content; TPC = total phenolic content.
DPPH = 2,2-diphenyl-1-picrylhydrazyl; IC_{50} = half-maximal inhibitory concentration
Solvant is expressed as fraction of solvents to water
Extraction Yield is expressed as 100 x (g dry extract/ g dry seaweed)
The IC_{50} of ascorbic acid as controls was 18.593 ± 0.135 µg/ml

3.2. Effect of solvent system in total phenolic and flavonoid content

Phenolic compound are secondary metabolites commonly found in plants and associated with several biological activities including protection from oxidative stress damage[30]. Several study reports the presence of thigh phenolic content in marine macro and microalgae in response to stress condition[31]. Table 1 shows the TPC of the extract measured by using the FC method. The TPC of the extract varied from 4.723 mg GAE/g to 36.738 mg GAE/g. The lowest TPC level produced by the usage of water as the extraction solvent. Contrast to the extraction yield result, extraction by using 100% acetone produced the highest TPC. The TFC by various solvent decreased in this following order: 100% acetone > 100% ethanol > 75% aqueous acetone > 75% aqueous methanol > 75% aqueous ethanol > 100% methanol >
50% aqueous ethanol > 50% aqueous acetone > 50% aqueous methanol > distilled water. Statistically, the TPC for extraction using 100% methanol, 75% and 50% aqueous ethanol were the same. Likewise, usage 75% and 50% aqueous methanol considered statistically the same. The significant highest TPC produced by the extraction using 100% acetone. Oppose to the extraction yield, the presence of water decrease the presence of phenolic content in the extracts. Our results showed that higher phenolic content was obtained with an increase in polarity of the solvent. Linear correlation showed the significant negative correlation of dielectric constant and TPC with correlation coefficient of 0.847. The results indicating that solvent polarity simultaneously and significantly correlated to TPC in inverse correlation. Increase of polarity would decrease the TPC. The similar results reported by Turkmen et al. in extraction of black and mate tea[14]. Zhou and Yu reported that 50% acetone extract contained the greatest level of total phenolic from wheat[32].

The TFC of the extract are reported in Table 1. The TFC of the extract varied from 4.848 mg QCE/g to 45.933 mg QCE/g. The lowest TFC produced by the extraction using distilled water, while the highest TFC produced by the extraction using 100% ethanol. The TFC decreased in this following order 100% ethanol > 100% acetone > 75% aqueous acetone > 75% aqueous methanol > 75% aqueous ethanol > 50% aqueous acetone > 50% aqueous ethanol > 1005 methanol > 50% aqueous methanol > distilled water. The TFC produced by 50% aqueous ethanol and 50% aqueous acetone were consider statistically the same. Regardless, all TFC were statistically different one another. It was observe that the effect of solvent of TFC is similar to the TPC. Extraction by 100% ethanol and 100% acetone produced the highest level of TFC and TPC. Linear correlation showed the significant negative correlation of dielectric constant and TFC with correlation coefficient of 0.554. Negative correlation revealed that increase of polarity inversely decrease the TFC. This result is similar to the extraction of phenolic from Limnophila aromatic guava and pisang mas, a variety of banana[15,33].

3.3. Effect of solvent system in antioxidant activity

Antioxidants are compounds which at low concentrations exhibit inhibition of the oxidation of proteins, carbohydrates, lipids and DNA. Some examples of antioxidant compounds are glutation, vitamins C and E, albumin, carotenoids and flavonoids. Antioxidants reduce cell damage caused by free radicals. Antioxidant intake can be obtained either from consuming fruits and vegetables due to high flavonoids or antioxidant supplements[34]. Phenolic compounds provide antioxidant activity through their activity as reducing agents that stop free radical chain reactions and chelate transition metals. This compound inhibits oxidation reactions by oxidizing itself. Oxidation reactions are crucial reactions and must occur in the body, but these reactions can also cause damage, so it is very important to increase the antioxidant complex system in the body[34]. The antioxidant activity of flavonoids is obtained from the hydroxyl group at the level of the conjugation between the flavonoid rings[35].

DPPH is a stable organic radical. DPPH absorption can be measures at 517 nm. DPPH will lose its absorption when accepting an electron from free radical species, resulting in visually noticeable colour change from purple to yellow, along with the decrease of absorbance value[36]. The IC\textsubscript{50} expresses the amount of antioxidant required to decrease the 50% of total DPPH concentration[37]. A lower IC\textsubscript{50} indicating the higher antioxidant activity of a compound[15]. Table 1 shows the IC\textsubscript{50} value in DPPH radical scavenging activity assay of the extract. It was found the extract of 100% ethanol produced the strongest antioxidant activity among other. The IC\textsubscript{50} value produced by 100% ethanol (13.603 μg/ml) is lower than ascorbic acid (18.593 μg/ml). Based on the classification of antioxidant activity by Phongpaicit, extracts of 100% ethanol, 75% aqueous acetone, 75% aqueous methanol, 100% acetone, 100% methanol, and 75% aqueous ethanol are classified as strong antioxidants with an IC\textsubscript{50} of 10 - 50 μg / ml[38]. The 50% aqueous ethanol and 50% aqueous methanol extracts are classified as mild antioxidants with an IC\textsubscript{50} of 50-100 μg / ml, while the 50% aqueous acetone and distiller water extracts are classified as weak antioxidants with 1IC\textsubscript{50}> 100 μg / ml. All results show a significant difference.

Linear correlation showed the significant positive correlation of dielectric constant and IC\textsubscript{50} with correlation coefficient of 0.797. Negative correlation revealed that increase of polarity would increase
the TFC as well. Extract produced by 100% ethanol exhibited the strongest antioxidant activity. This result correlated with high presence of TFC and TPC. Linear regression correlation between IC\textsubscript{50} and both TPC and TFC produced positive coefficient correlation of 0.813 and 0.469, respectively, indicating that either TPC or TFC simultaneously and significantly correlated to IC\textsubscript{50}. IC\textsubscript{50} was highly correlated to TPC than TFC. Phenolic were the main antioxidant components and their level were directly proportional to antioxidant activity. The similar result reported by Do et al. [37].

4. Conclusion
The antioxidant activity of bulung sangu was evaluated using DPPH radical scavenging assay. In general, extraction yield increased with the presence of water. Solvent system consisted of water and organic solvent showed higher extraction yield as compared to either water or organic solvent without combination. This may be due to the ability of combination solvent to facilitate the isolation of varied polarity compound. In contrast to extraction yield, TPC, TFC and antioxidant activity decreased with the presence of water in solvent system. Usage of pure organic solvent produced higher TPC, TFC, and antioxidant activity. As compared to methanol, pure methanol and ethanol produced high level of TPC and TFC, likewise resulted in high antioxidant activity. Pure acetone produced highest level of TPC, while pure ethanol produced highest level of TFC and antioxidant activity. Nevertheless, extraction by 100% ethanol produced relatively low extraction yield. By considering TPC, TFC and antioxidant activity, extraction by using 100% ethanol considered the most effective. The results of this work indicating that a proper extraction solvent for bulung sangu produced a potential medicine against free-radical-associated oxidative damage.

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