

## ULTRASONIC-ASSISTED EXTRACTION OF ANTIOXIDANT PHENOLIC COMPOUNDS FROM *EUCHEUMA COTTONII*

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### Abstract

*The influences of ultrasonic-assisted extraction (UAE) conditions on phenolic compounds of Eucheuma cottonii seaweed were investigated. The effects of temperature, time and liquid-solid ratio were evaluated based on the total phenolic content (TPC). The result showed a significant variability of effect of temperature, time, and liquid-solid ratio on TPC. Application of ultrasound reduced the optimum temperature, time and liquid-solid ratio of the extraction when compared to the conventional extraction. The highest phenolic content (4.44 mg gallic acid equivalent/g dry weight) was obtained at 3:1(v/w) of liquid-solid ratio, 55°C of temperature and 35 min of extraction time. At this condition, the extract showed ability to scavenging 91% of 1,1-diphenyl 2 picrylhydrazyl (DPPH radical) and 10.9% of H<sub>2</sub>O<sub>2</sub>. High ability of the extract to scavenge DPPH radical but low in scavenging H<sub>2</sub>O<sub>2</sub> indicated that the extract dominated by primary antioxidant compounds which work by donating hydrogen atoms to bind free radical.*

**Keywords:** *antioxidant; DPPH radical; Eucheuma cottonii; H<sub>2</sub>O<sub>2</sub>; phenolic compounds; ultrasonic-assisted extraction*

### Abstrak

**EKSTRAKSI BERBANTU ULTRASONIK SENYAWA FENOLIK ANTIOKSIDAN DARI *EUCHEUMA COTTONII*.** Penelitian ini mempelajari pengaruh kondisi operasi ekstraksi berbantu gelombang ultrasonik terhadap perolehan senyawa fenolik *Eucheuma cottonii*. Peubah-peubah yang dipelajari meliputi pengaruh suhu, waktu dan rasio cairan-padatan terhadap kadar fenol total (TPC) pada ekstraksi berbantu gelombang ultrasonik. Hasil penelitian menunjukkan bahwa pengaruh peubah-peubah operasi tersebut cukup beragam. Pemaparan gelombang ultrasonik pada proses ekstraksi tersebut terbukti menurunkan suhu, waktu dan rasio cairan-padatan yang optimum disbanding dengan metode ekstraksi konvensional. Kadar senyawa fenolik tertinggi (4,44 mg ekuivalen asam galat/g berat kering) dicapai pada ekstraksi menggunakan rasio cairan-padatan 3:1(v/w) pada suhu 55°C selama 35 menit. Ekstrak yang diperoleh pada kondisi ini menunjukkan kemampuan antioksidasi melawan 1,1-diphenyl 2 picrylhydrazyl (radikal DPPH) dan hidrogen peroksida, masing-masing sebesar 91% dan 10,9%. Perbedaan yang mencolok dalam aktivitas antioksidasi ini menunjukkan bahwa ekstrak *E. cottonii* didominasi oleh senyawa antioksidan primer yang bekerja dengan menyumbangkan atom hidrogen untuk mengikat radikal bebas.

**Kata kunci:** *antioksidan; radikal DPPH; Eucheuma cottonii; H<sub>2</sub>O<sub>2</sub>; senyawa fenolik; ekstraksi berbantu gelombang ultrasonik*

### INTRODUCTION

Many synthetic antioxidants, such as butylated hydroxyl toluene (BHT) and tert-butyl hydroxyl quinone (TBHQ) have been reported to have potential health hazards. These antioxidants have been restricted for use in foods as they are suspected to be

carcinogenic. Hence their use as food additives is under strict regulation in many countries. These conditions, together with recent consumers' interests in natural product, have driven the search of natural antioxidant sources as alternatives to replace synthetic

antioxidants in preventing oxidation in complex food systems (Romero *et al.*, 2004).

Owing to their high polyphenol content, macro algae are gaining interest as potential natural sources of antioxidant compounds from marine plant. Methanol extracts of red and brown algae had been identified to contain a series of polyphenolic compounds such as catechins (e.g. gallic acid, epigallocatechin gallate), flavonols and flavonol glycosides. These compounds demonstrated a great capacity in capturing free radicals which cause oxidative stress and helping in preventing some types of cancers (Wang *et al.*, 2009). *Kappaphycus alvarezii* (Doty) Doty ex P.C. Silva also known as *Eucheuma cottonii* belongs to a red algae has been cultured and utilized for source of traditional remedies as well as food materials in Asian countries, such as carrageenan and antioxidant (Wang *et al.*, 2009). The algae powder showed ability to decrease total cholesterol, low density lipoprotein (LDL), triglyceride, and increase the level of high density lipoprotein (HDL) and superoxide dismutase (SOD) status in the liver tissues of hypercholesterolemic rats (Wresdiyati *et al.*, 2008). Kumar *et al.* (2008) reported that the total phenol content of various solvent extracts of *K. alvarezii* varied from 0.683 to 2.05%. Among the extracts obtained, the methanol extract showed the highest chelating activity and reducing power. Moreover, their reducing power and hydroxyl radical scavenging activities of *Kappaphycus* were higher compared to  $\alpha$ -tocopherol.

Previous researches applied maceration in extracting phenolic compounds of *E. cottonii* (Damongilala *et al.*, 2013). Maceration is a simple extraction method which requires a long extraction time and more consumption of solvent. In addition, most active ingredients of plants are found in the *E. cottonii* cells, making it difficult for mechanical crushers to break cells for extraction, while chemical crushing methods may damage the active molecules and inactivate the extract (Lai *et al.*, 2013). Developing a fast and efficient way for production a high quality of botanical products has become an issue of concern in pharmaceutical and food industries to overcome the problem of the existing methods.

Much attention has been given to apply ultrasound for the extraction of natural products. Compared to conventional extraction, ultrasound-assisted extraction (UAE) permits to achieve complete extraction in shorter time, reduce solvent consumption, increase yield of extracted components and increase rate of extraction. The enhancement of UAE efficiency is attributed to a phenomenon of cavitation bubbles produced in the solvent as a response to an ultrasonic wave. During exposure to the wave, the cavitation bubbles are able to grow and finally collapse when they reach a critical point. The implosion of cavitation bubbles generates macro-turbulence, high-velocity inter-particle collisions and perturbation in micro-porous particles of solid

material which accelerates internal diffusion. When these bubbles collapse onto the surface of a solid material, the bubbles release micro jets directed towards solid surface that result in surface peeling, erosion and particle breakdown which allowing higher penetration of the solvent into solid particles and the intracellular product compounds released by disrupting the cell walls. A great performance was reported in application of UAE to extract herbal, oil, protein and bioactive and functional compounds e.g. polyphenolics, anthocyanins, aromatic compounds, polysaccharides from variety of matrices such as plant and animal materials. Moreover, it was reported that applications of UAE improved the extraction yield of bioactive compounds by 6-35% compared to conventional process (Chemat *et al.*, 2011; Vilku *et al.*, 2008).

In general, solvent type, solid to solvent ratio, time, temperature and ultrasound power are influential parameters for extracting phenolic compound by using UAE. Unfortunately, study of UAE application for extracting phenolic compounds of *E. cottonii* has not been explored. Hence, this study was aimed to determine the effect of UAE parameters on antioxidant compounds of *E. cottonii*. The antioxidant extracts were determined as total phenolic content, ability to scavenge free radicals of DPPH radical and activity to scavenging  $H_2O_2$ .

## MATERIALS AND METHODS

### Materials

Fresh *E. cottonii* was obtained from Karimun Java (Central Java, Indonesia). The plant was dried at 55°C for 48 hour, and ground into powder. Folin Ciocalteu Reagent (FCR),  $Na_2CO_3$  (99%), gallic acid (99%), and methanol (90%) were purchased from Merck, while 1,1 Diphenyl 2-picrylhydrazyl (DPPH radical, 90%) and  $H_2O_2$  were obtained from Sigma Aldrich.

### Ultrasonic-Assisted Extraction

One gram of *E. cottonii* powder was mixed with 10 mL of methanol in 100 mL of volumetric flask and covered with aluminum foil. The extraction flask was then placed in an ultrasonic bath (Krisbow DSA50-GL). The extractions were set for various temperatures (35-60°C), times (10-45 min) and ratio of liquid-solid (3:1-10:1 v/w). After the extraction, the extract was filtered and analyzed for antioxidant activities.

### Total Phenolic Content (TPC)

The total phenolic content of the extracts was determined using the method of Waterhouse (1999). Briefly an aliquot of the sample (0.2 mL), distilled water (15.8 mL) and FCR (1 mL) was placed in capped test tubes. After addition of 10 mL of sodium carbonate (20% w/w), the tubes were vortexed for 10 min then allowed the mixture to react at room temperature for 2 hours. The absorbance of the

mixture was measured at 765 nm using spectrophotometer (Optima SP 300, Japan) compared to the blank. The amount of total phenolic content was expressed as gallic acid equivalents (mg GAE/g dry weight).

### 1,1 Diphenyl 2-picrylhydrazyl Scavenging Activity (DPPH-SA)

The DPPH-SA assay was carried out following the procedure of Banerjee *et al.* (2005). An aliquot of extract (1.0 mL) was added to solution of DPPH radical (3 mL, 0.1 mM) freshly prepared, and then kept the mixtures in the dark for 30 min. The absorbance of the mixture was read using a spectrophotometer at 517 nm (Optima SP 3000, Japan). Activity of the extract to DPPH-SA was expressed as:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}} \times 100\% \quad (1)$$

### Hydrogen Peroxide Scavenging Activity (HP-SA)

The HP-SA assay was carried out base on the procedure of Ruch *et al.*, (1989). A solution of H<sub>2</sub>O<sub>2</sub> (43 mM) was mixed with phosphate buffer (0.1 M, pH 7.4) in ratio 1:4 (v/v). An aliquot of extract (1.0 mL), a solution of phosphate buffer (2.4 mL, pH 7.4, 0.1 M) and a solution of H<sub>2</sub>O<sub>2</sub>-phosphate buffer (0.6 mL) were placed in test tube. The absorbance of the mixture was read at 230 nm using spectrophotometer (Optima SP 300, Japan) after 40 min incubation at dark room. The H<sub>2</sub>O<sub>2</sub>-phosphate solution was used as the blank. The HP-SA was determined as:

$$\text{H}_2\text{O}_2 \text{ scavenging effect (\%)} = \frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}} \times 100\% \quad (2)$$

## RESULTS AND DISCUSSION

In this study, effects of temperature, time and solvent-solid ratio of UAE on antioxidant activity of *E. cottonii* were studied. All the obtained extracts were measured for TPC. The extracts of solvent-solid ratio were subsequently determined for DPPH-SA and HP-SA.

### Effect of UAS Temperature on TPC

Figure 1 shows the gradual increase of TPC in line with the increase of temperature from 35 to 55°C. The results showed that the solubility and diffusion of phenolic compounds from *E. cottonii* matrix particles into the extraction medium increased with temperature. The increase of extraction temperature allowed permeability of cell wall to increase while viscosity of solvent decrease. This increased diffusivity of the solvent in the solid matrix and the solubility of the phenolic compounds in the solvent, which lead to more compounds extracted. Moreover, the decrease of the solvent viscosity permitted to reduce tensile surface and encouraged more cavitations intensity which in turn extracted higher phenolic compounds and accelerated UAE. Similar trends in the relationship of TPC and extraction temperature have been reported by Liu *et al.* (2010)

who studied the chlorogenic acid of *Folium eucommiae*.

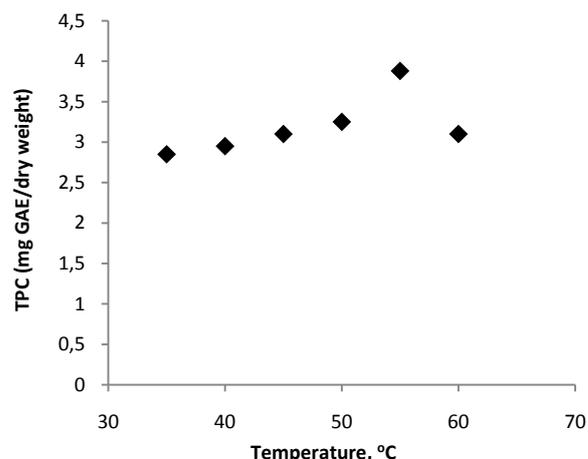


Figure 1. Effect of UAE temperature on the total phenolic content (TPC) of red algae *E. cottonii*

This study showed the TPC of red seaweed reached the maximum value (3.88 mg GAE/g dry weight) at 55°C of UAE. Heo *et al.* (2005) found 70°C as the best extraction temperature of the antioxidant compound of various seaweeds. At this temperature they obtained 1.04-109.36 mg/g dry weight sample of the phenolic concentration after extraction for 24 h, depend on the seaweed species. The different in the best extraction temperature indicated that application of ultrasound reduced the optimum extraction temperature of the phenolic compared to the conventional extraction. Indirect heat transfer process was observed in the conventional method where temperature gradient was the driving force. In this process, the heat gradually propagates from solvent to the solid. Meanwhile, direct contact between solid and ultrasonic wave in UAE allow achieving lower optimum temperature compared to the conventional extraction. Ultrasound aids a mechanical effect by continuous compression and evacuation cycle allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between the solid and liquid phase, as a result the solute quickly diffuses from the solid phase to the solvent (Rostagno *et al.*, 2007). Phenolic compounds were unstable in higher temperature. Therefore, high extraction temperature might enhance extract impurities due to co-extraction of other undesirable compounds (Liu *et al.*, 2010).

It should also be noted that although increasing temperature could improve the TPC, improvement of temperature beyond a certain value can lead to decomposition of some phenolic compounds. In this study, exposure the seaweed at higher extraction temperature than 55°C allowed the phenolic compounds to be decomposed during UAE thus reduced TPC. Therefore, it is important to select the correct range of extraction temperature to be studied in order to obtain the best antioxidant performance.

### Effect of UAS Time on TPC

TPC increased along with the extension of the extraction time (Figure 2). The results showed 35 min as the optimum extraction time of TPC of *E. cottonii*, which obtained 4.2 mg GAE/g dry weight. Solid-liquid extraction is a mass transport phenomenon in which compounds in solid matrix migrate into solvent by diffusion and osmotic mechanism which induced by ultrasounds. Application of ultrasound in solid-liquid extraction forms cavitations bubbles which break the plant cells to facilitate penetration of solvent into the cells. The penetration facilitates swelling and hydration, causing an enlargement in the pores of the cell wall, which improves the diffusion process and leading to enhance mass transfer (Bilgin and Shahin, 2013). This result agreed that extraction time is an important variable in UAE of phenolic compounds from plant materials. A longer extraction time permits more contact time for the cavitation bubbles to rupture more plant cells, result in an increase of the TPC extracted (Wang *et al.*, 2012). The result was in accordance with Han *et al.* (2012) who reported the improvement of phenolic of Betel nut seed with time by using UAE method, respectively.

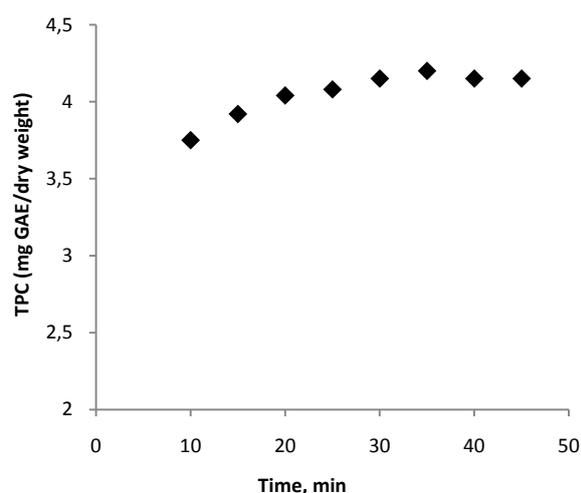


Figure 2. Effect of UAE time on the total phenolic content (TPC) of red algae *E. cottonii*

However, Chew *et al.* (2011) found that prolonging extraction time no longer affect the phenolic level of *Orthosiphon stamineus* significantly when the diffusion process had reached equilibrium. Figure 2 shows TPC no longer significantly improved when extraction time was longer than 35 min. Extension of extraction time over the equilibrium time leads the phenolics to be exposed to light and oxygen in longer period. This exposure allowed the phenolic compounds to be oxidized which in turned decrease the obtained TPC. Moreover, prolonging the extraction time may cause accumulation of compounds that could promote oxidation (Bazykina *et al.*, 2002). This research showed that UAE for over 35

minutes could lead the phenolics of *E. cottonii* to be oxidized.

The mechanism of UAS kinetic, as showed by Figure 2 is in agreement with the result of Bilgin and Shahin (2013). They proposed that UAS mechanism has 2 stages of extraction. At the beginning of the extraction, dissolution of soluble components on surfaces of the plant matrix occurs. This first stage results in a rapid increase of TPC. The next stage is mass transfer of the solute from the plant matrix into the solvent by diffusion and osmotic processes, which is slower process. In this study, slightly raise of TPC was observed over 35 min. Therefore, 35 min was accepted as the optimum time in this process.

The maximum TPC obtained in this research (4.40 mg GAE/g dry weight) was lower than TPC of Damongilala *et al.* (2013) who obtained 5.87 mg GAE/g dry weight for extracting phenolic of *E. cottonii*. However, Damongilala *et al.* (2013) conducted the study by maceration for  $3 \times 24$  hour compared to this study by using UAS for 35 min only. Hence, this study confirmed that UAS reduce the extraction time. This study showed higher result of TPC than report of Chew *et al.* (2008) who found TPC of *E. cottonii* was  $115 \pm 35$  mg GAE/100 g dry weight after 1 hour maceration.

### Effect of Liquid-Solid Ratio on TPC

Effect of solvent-solid ratio of UAE on TPC is presented in Figure 3. The highest TPC (4.440 mg GAE/g dry weight) was obtained at 3:1 of the liquid-solid ratio. However, there was insignificant change of TPC obtained at solvent-solid ratio studied (3:1-10:1, v/w). Result of this study was in line with other UAE reports. Oancea *et al.* (2013) also reported insignificant different of antocyanin of black gold sweet cherry at 10:1-20:1 (v/w) for 20 min by using UAE. Petigny *et al.* (2013) reported insignificant effect of solid-solvent ratio at 4-10% on yield of UAE Boldo leaf.

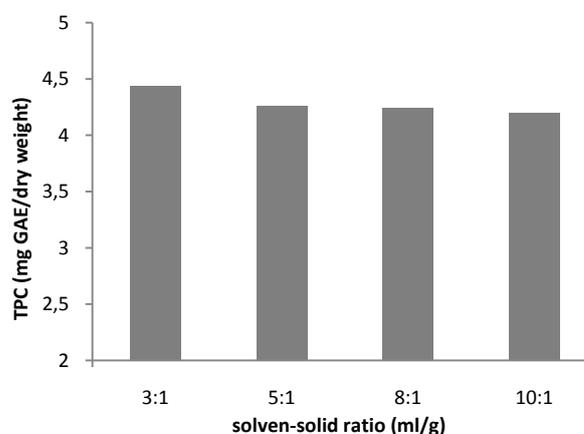


Figure 3. Effect of UAE solvent-solid ratio (v/w) on the total phenolic content (TPC) of red algae *E. cottonii*

In conventional extraction techniques such as maceration, higher volume of solvent will increase the extract yield but too much solvent is inefficient. In UAE, the ultrasound wave creates frictions between solid samples. The frictions produce heat and allow cavitation bubbles to damage the cell wall and accelerate the extraction of the phenolic compounds. Addition of solvent volume cause more distance between the solid particles thus reduce the frictions (Tcholakova *et al.*, 2008). This result showed application of UAE allowed to apply less solvent, which in turn reduced cost in extraction process.

#### Effect of Liquid-solid Ratio on DPPH Radical Scavenging Activity (DPPH-SA)

A 1,1-diphenyl-2-picrylhydrazyl (DPPH) compound is a stable free radical that possesses a nitrogen free radical that is commonly used to measure the antioxidant activity of tissue extracts. The free radical is unpaired electron which unstable and highly reactive molecule. Scavenging activity of DPPH radicals by the antioxidant occurs through proton donation to the radical. Compound that is able to donate its proton shows strong radical scavenging activity. The phenolic compounds release their hydrogen atoms to bind to the 1,1-diphenyl-2-picrylhydrazyl and subsequently change into 1,1-diphenyl-2-picryl hydrazine non radical. The latest formed is stable and do not attack other molecules to form a new radical. An increasing number of 1,1-diphenyl-2-picryl hydrazine non radicals will be marked with changing color from purple to yellow.

The DPPH-SA of the extracts in various solvent-solid ratios are presented in Figure 4. This figure shows increasing solvent volume reduced the ability of extract to scavenge radical DPPH. The highest DPPH-SA was obtained at 3:1 solvent-solid ratio which was around 91%. This trend was in line with the result of TPC (Figure 3). The higher content of TPC implied the extract contained more hydrogen atoms to bind more DPPH radicals due to increasing number of DPPH radicals stabilized by hydrogen atom donors derived from the phenolic compounds and turn them into non-radical compounds. Previous studies have shown that phenolic compounds are the main contributors on the antioxidant activity of various seaweeds. A positive correlation has been documented between TPC and antioxidant activities of different seaweed extracts by many researchers (Wang *et al.*, 2009). However, these antioxidant assays were not specific to any particular antioxidants. This result showed although the TPC content slightly improved, however, the DPPH-SA significantly rose. The result suggested that the extract could also contain other compounds than phenolics which also have strong radical scavengers. Souza *et al.* (2012) reported red seaweed contains galactose, 3,6-anhydrogalactose and 6-O-methylgalactose which are sulfate polysaccharides and showed ability to inhibit DPPH radical. Chew *et al.* (2008) who studied antioxidant

activity of three seaweeds found *K. alvarezzi* showed higher activity in scavenging DPPH radical than *C. racemosa*, although TPC of the later seaweed was higher that of *K. alvarezzi*.

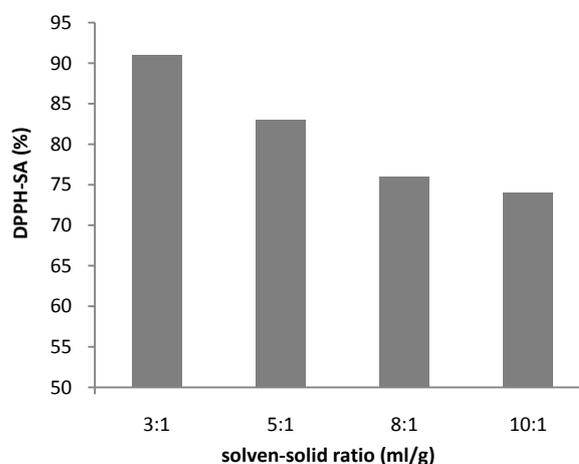


Figure 4. Effect of UAE solvent-solid ratio (v/w) on DPPH radical scavenging activity (DPPH-SA) of red algae *E. cottonii*

Although lower in TPC, the DPPH-SA result of this study was higher than the result of Damongilala *et al.* (2013) who showed only 69% as the best DPPH-SA. This higher of DPPH-SA value indicated that shorter extraction time allowed protecting the antioxidant compounds of the extract from exposed to environments including oxygen and light which was trigger to oxidation during the extraction process, as discussed at Effect of UAS Time on TPC

#### Effect of Liquid-Solid Ratio on H<sub>2</sub>O<sub>2</sub> Scavenging Activity (HP-SA)

This analysis of antioxidant performance is base on the phenomenon of transition metals such as Fe<sup>2+</sup> or Cu<sup>+</sup> which catalyze formation of the hydroxyl radical from H<sub>2</sub>O<sub>2</sub> in the body through non enzymatic Fenton reaction. H<sub>2</sub>O<sub>2</sub> is a weak oxidizing agent, not a free radical. Moreover, H<sub>2</sub>O<sub>2</sub> is also the precursor of hypochlorous acid (HOCl), a powerful oxidizing agent. Scavenging of H<sub>2</sub>O<sub>2</sub> by antioxidants is essential for the body's defense mechanisms to inhibit free radical formation caused by Fenton reaction (Smith *et al.*, 2004). The antioxidant that scavenging non radical such as H<sub>2</sub>O<sub>2</sub> known as secondary antioxidant which offers their antioxidant activity by deactivating the active species and possible precursors of free radicals (Yanishlieva, 2001).

The scavenging ability of phenolic compounds of *E. cottonii* on H<sub>2</sub>O<sub>2</sub> is presented in Figure 5. This figure shows the more solvent applied, the less HP-SA of the extract observed. Figure 5 shows a similar trend of liquid-solid ratio on HP-SA with that on DPPH-SA. Although the extract demonstrated high ability to bind DPPH radicals (91%), the maximum HP-SA of the extract was only 10.95% which obtained at 3:1 of liquid-solid ratio. Similar result was reported by Pise

*et al.* (2010) who found HP-SA of the phenolics from *Porphyra indica* seaweed was only 10% after 24 h maceration. This result was lower than HP-SA of BHA which was 44.58%.

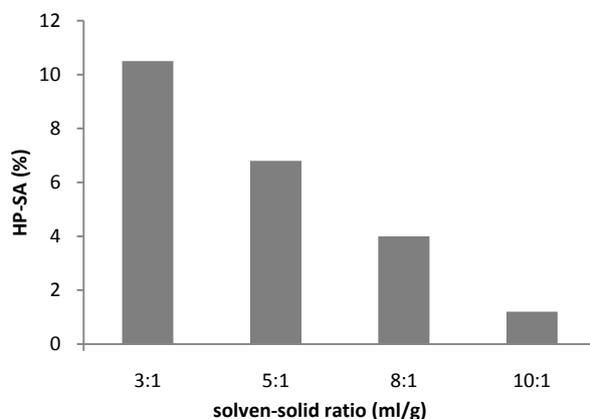


Figure 5. Effect of UAE solvent-solid ratio (v/w) on  $H_2O_2$  scavenging activity (HP-SA) of red algae *E. cottonii*

A significant different ability of the extract toward DPPH-SA and HP-SA suggested that the extract contained mostly primary antioxidant compounds rather than secondary ones. Primary antioxidant works as radical chain breaker by donating hydrogen atoms to bind radical and convert it into stable molecule (Frankel, 2005). High value of DPPH-SA indicated that the extract comprised of hydrogen atoms which are able to scavenging up to 91% of radical DPPH. This result was in line with Wang *et al.* (2009) who postulated that the major role of algal antioxidants is a radical scavengers and primary chain-breaking antioxidants. Hence, it was suggested that the extract contained minor amount of secondary antioxidant compounds that work as non radical inhibitor which will react with  $H_2O_2$ . This low in secondary antioxidant content resulted in low ability to scavenging  $H_2O_2$ .

## CONCLUSION

This study showed that the maximum TPC of seaweed *E. cottonii* obtained by UAE at 55°C, 35 min and 3:1 (v/w) solvent-solid ratio was 4.44 mg GAE/g dry weight. The TPC of the extract had a positive correlation with scavenging DPPH radical, which maximum was 91%. Regardless, positive correlation between TPC and HP-SA, the extract only showed just above 10% on scavenging  $H_2O_2$ . Hence, it was suggested that the extract comprised of primary antioxidant as a major antioxidant compounds which work by binding free radical. This study confirmed that the antioxidant assay could have a great effect on determining antioxidant effectiveness. It requires further studies to understand the detail antioxidant compounds of *E. cottonii* and their action in performing antioxidant on different antioxidant assays.

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