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Antioxidant Activity of Brown Algae *Sargassum species* Extract from the Coastline of Java Island

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ABSTRACT

The coastlines with rocky shores at Java Island Indonesia, Gunung Kidul (Yogyakarta) and Jepara (Central Java), have abundant resources of brown algae *Sargassum* sp. Little effort, however, has been made to explore the antioxidant potential of the algae harvested from these area. Tropical brown algae have proven to produce a very effective antioxidant defence system due to the strong UV radiation in the tropical environment. The Total Phenolic Content (TPC) of the extracts were evaluated by using Follin Ciocalteau reagent, while antioxidant activities were evaluated by using 2,2-Diphenyl-1-Picrylhydrzyl Radical Scavenging Activity (DPPH-RSA) and Ferrous Ion-Chelating (FIC) ability. The effects of treatments on TPC and antioxidant activities were analysed using analysis of variance models. There were four treatments and three replicates. These treatments were extract types (cytoplasmic and membrane bound), harvest sites (Gunung Kidul and Jepara), harvest seasons (dry and rainy) and various *Sargassum species*. The TPC of extracts varied from 0.006-0.65 and 6.72-21.99 g phloroglucinol equivalent/100 g dried extract for cytoplasmic and membrane bound extract, respectively. The DPPH-RSA in extracts concentration of 0.45 mg mL^{-1} were in the range of 14.61-48.71 % for membrane bound and 0.17-44.05 % for cytoplasmic extracts. The FIC in extract concentration of 3.3 mg mL^{-1} were in the range of 2.0512.51 and 26.77-68.80 % for cytoplasmic and membrane bound extract, respectively. Nevertheless, these extracts had lower activity when compared with BHT and EDTA as positive control of RSA-DPPH and FIC, respectively. The antioxidant activities of *Sargassum* were influenced by types of extracts, harvest sites, seasons and species. The antioxidant activity of membrane bound was higher than cytoplasmic extract. The increasing of DPPH-RSA and FIC were proportional to TPC. The highest antioxidant activity was found at *S. hystrix* from Gunung Kidul area harvested during dry season.

Keywords: *Sargassum Species*, Java Island, Antioxidant Activity, Total Phenolic Content

1. INTRODUCTION

The genus *Sargassum*, a kind of brown algae, is a tropical and sub-tropical brown seaweed in subtidal and intertidal areas, comprising 150 species (Olabarria *et al*., 2009). The distribution and population structure of *Sargassum species* were influenced by water temperature, tidal levels, water movement and substrate types (i.e., rocky shores) (Ang, 1986; Ateweberhan *et al*., 2005). Indonesia is a tropical country that has two seasons, rainy and dry season. The coastlines with rocky shores at Java Island Indonesia, Gunung Kidul (Yogyakarta) and Jepara (Central Java), have abundant resources of seaweed, especially brown algae *Sargassum* sp., however little effort has been made to explore the antioxidant potential of seaweed harvested from these area. *Sargassum* had been studied extensively showing high antioxidant potential in vitro (Lim *et al*., 2002; Santoso *et al*., 2004; Kim *et al*., 2005; Park *et al*., 2005; Cho *et al*., 2007; Zubia *et al*., 2007; 2008; Budhiyanti *et al*., 2011). Antioxidant compounds play an important role against various diseases (e.g., atherosclerosis, chronic inflammation, cardiovascular disorders and cancer) and aging processes (Kohen and Nyska, 2002).

Tropical macroalgae are expected to develop a very effective antioxidant defence system due to the strong UV radiation in the tropical environment (Zubia *et al*., 2007; Matanjun *et al*., 2008). In fact, previous studies have demonstrated that UV radiation induces the promotion of antioxidant defence in macroalgae (Aguilera *et al*., 2002; Bischof *et al*., 2002).

The phenolic compounds were one of the most effective antioxidant in brown algae (Nagai and Yukimoto, 2003). In general, the phenolic content of brown algae was 20-30% dry weight (Ragan and Glombitza, 1986). Many studies have shown that phlorotannins were the main phenolic compounds detected in brown algae (Koivikko, 2008). Phlorotannin is a group of phenolic compounds which are formed by the polymerization of phloroglucinol (1, 3, 5 trihydroxybenzene) monomer units and synthesized in the acetate-malonate pathway in marine alga (Ragan and Glombitza, 1986; Arnold and Targett, 2000). Koivikko *et al*. (2005) divided phlorotannin into three parts, soluble phlorotannin from algal matrix or cytoplasmic phlorotannin, cell-wall bound phlorotannin that attached to the membrane or cell wall and exuded phlorotannin that exude into the surrounding seawater. Antioxidant activity from membrane bound extract was higher than cytoplasmic extract (Budhiyanti *et al*., 2011).

The northern coast of Java (Jepara) and the south coast of Java (Gunung Kidul) were selected for this study due to significant different in environmental condition, geographical and hydrodynamic features of two sites. The Jepara coast has low and calm waves, *Sargassum* is abundant in both dry and rainy season. Gunung Kidul coast, on the other hand, has high waves and *Sargassum* declines in biomass during the rainy season. Gunung Kidul has longer emersion cycle than Jepara, therefore *Sargassum species* at Gunung Kidul has longer exposed to solar radiation. Levels of phenolic compounds can be increased by excessive algae exposure to UV radiation (Henry and Alslyne, 2004; Diaz *et al*., 2006).

There is no information about the influence of environmental factors in antioxidant activity of *Sargassum* in Java Island, Indonesia. The objectives of this study were to study the effects of extract type, harvest site and season on antioxidant activity of various *Sargassum species*. In addition to total phenolic content, the antioxidant activity of crude extracts were studied using 2,2-diphenyl-1-picrylhydrzyl (DPPH) radical scavenging activity and ferrous ion-chelating ability.

2. MATERIALS AND METHODS

2.1. Plant Material

The brown algae *Sargassum* were collected from the intertidal rocky shore of Gunung Kidul (Yogyakarta area, 8°8'1" S; 110°33'16" E) and Jepara (Central Java area, 6° 36' 58.60" S, 110° 38' 50.75" E) on dry (April 2010) and rainy (October 2009) season.

The taxonomical identification of specimens were confirmed at Plant Taxonomy Laboratory, Faculty of Biology, Gadjah Mada University. There were 3 species from Jepara that were taken during dry and rainy season, *Sargassum polyceratium* (SP), *S. angustifolium* (SA) and *S. filipendula* (SF) and 3 species from Gunung Kidul that were taken during dry season (*S. hystrix* (SH), *S. siliquosum* (SS) and *S. mcclurei* (SM)) and 2 species in rainy season (*S. hystrix* (SH) and *S. cinereum* (SC)).

The algae were sorted out to remove epiphytes and washed with water to remove sand. The washed seaweed was stored at -20°C until used.

2.2. Preparation of Seaweed Extracts

Crude extracts of *Sargassum* sp. were prepared using the modified method from Lim *et al*. (2002); Haider *et al*. (2009); Ye *et al*. (2009) and Budhiyanti *et al*. (2011). Ten grams of dried seaweed were immersed in 100 mL methanol and shaked for 1 h at 40°C, followed by centrifuging the extract to collect the supernatant. The extraction was repeated three times. The supernatant were transferred to a conical flask and then washed with chloroform in a separatory funnel to remove pigments. The extracts were evaporated under low pressure until all solvent had evaporated. The extracts were called as cytoplasmic extract. The residues from the extractions of cytoplasmic extract were dried in oven (30 min, 60°C). The dried residue (200 mg) were extracted with 8 mL of 1 M sodium hydroxide (NaOH), stirred for 2 h and neutralized with H_3PO_4 (Koivikko *et al*., 2005; Koivikko, 2008). The extracts were called as membrane bound extract. The extract was evaporated and freeze dried, then dissolved in distilled water. They were kept in the dark and stored at 4°C prior analysis. The cytoplasmic and membrane bound extracts were determined total phenolic compound, free radical scavenging activity and ferrous ion chelating ability.

2.3. Total Phenolic Content (TPC)

Total Phenolic Content (TPC) was determined spectrophotometrically by Follin-Ciocalteau method according to the method of Chandini *et al*. (2008). One hundred µL aliquot of samples were mixed with 2 mL of 2% sodium carbonate solution. After 2 min, 100 µL of 50% Folin-Ciocalteau's phenol reagents were added. The mixtures were allowed to stand for 30 min at ambient temperature in the dark and the absorbances were measured at 720 nm. Total content of phenolic compounds were calculated based on a standard curve of phloroglucinol. The curve was made by plotting concentration (mg mL^{-1}) versus

absorbance (nm), with regression equation of standard curve y = 3.910x+0.002, R^2 = 0.99, where x = concentration and y = absorbance. The phenolic content was expressed as g of Phloro Glucinol Equivalents (PGE) per 100 g of dry extract (Zubia *et al*., 2007). This analysis was made in three replications for each extract.

2.4. Free Radical Scavenging Activity (RSA)

The free Radical Scavenging Activity (RSA) was determined according to the method of Chandini *et al*. (2008) with slight modification. One millilitre of seaweed extracts in methanol with concentration of 0.45 mg mL^{-1} was mixed with 2 mL of 0.08 mM methanolic solution of DPPH. The mixtures were then vortexed, left for 30 min at room temperature in the dark and the absorbances were measured at 517 nm. BHT was used as the positive control. As a blank was one millilitre of methanol mixed with 2 mL of 0.08 mM methanolic solution of DPPH. Radical scavenging activity was calculated using the equation:

Radical Scavenging Activity (%)
= [1 - (A sample/A blank)×100%]

2.5. Ferrous Ion-Chelating Ability (FIC)

The Ferrous Ion-Chelating (FIC) ability was determined according to the method of Ye *et al*. (2009) and Wang *et al*. (2009). One millilitre of seaweed extract solutions (with concentration of 3.33 mg mL^{-1}) were mixed with 0.05 mL of 2 mM $FeCl_2$, 0.2 mL of 5 mM ferrozine and 2.75 mL distilled water. The mixtures were shaken at room temperature in the dark for 10 min and the absorbances of the iron ions-ferrozine complex were measured at 562 nm. EDTA was used as the positive control. The results were expressed in percentage of chelating ability (% chelating ability), using following equation:

Ferrous ion-chelating ability (%) =
[1-(A sample- A blank)/ A control]×100%

$FeCl_2$ solution substituted by distilled water was used as a blank and the sample substituted by distilled water was used as negative control.

2.6. Statistical Analysis

The effects of treatments on total phenolic contents and antioxidant activities were analysed using Analysis of Variance (ANOVA) and carried out in three replicates. The treatments were extract types (2 levels: cytoplasmic and membrane bound extract), harvest sites (2 levels: Gunung Kidul and Jepara), harvest seasons (2 levels: dry and rainy) and various *Sargassum species* (nested within extract, season and site). The species were nested because they could not be found in all seasons. The data were recorded as mean ± Standard Deviation (SD). The p values that less than 0.0001 were considered significant. The linier regression analysis, correlation coefficients and determination coefficients were determined between total phenolic content and % radical scavenging activity; total phenolic content and ferrous ion-chelating ability.

3. RESULTS

3.1. Total Phenolic Content (TPC):

The Total Phenolic Content (TPC) of the extracts were determined from regression equation of standard curve y = 3.91x+0.002 and expressed as gram of Phloro Glucinol Equivalents (PGE) per 100 g of extract. The amount varied from 0.006-0.65 g PGE/100 g extract for cytoplasmic and 6.72-21.99g PGE/100 g extract for membrane bound extract. There was significant interaction ($p<0.0001$) on species that nested within (extract x season x site) which means that total phenolic content was influenced by types of extracts, seasons, sites and species (**Table 1 and Fig. 1**). The TPC revealed that membrane bound extracts of *S. hystrix* from Gunung Kidul area at dry season showed the highest yield among the other extracts.

3.2. Radical Scavenging Activity (RSA)

The parameter used to measure the free radical scavenging activity of the extract was % radical scavenging activity of DPPH (% DPPH-RSA). The presented data in **Fig. 2** indicated that RSA in extract concentration of 0.45 mg mL^{-1} were in the range of 14.61-48.71% for membrane bound and 0.17-44.05% for cytoplasmic extracts. Nevertheless, the results of these extracts had lower antioxidant activity than the commercial antioxidant BHT (100 ppm, 89.83%).

There was significant interaction ($p<0.0001$) on species that nested within (extract x season x site) which means that % RSA was influenced by types of extracts, seasons, sites and species (**Table 1 and Fig. 2**). The % RSA revealed that membrane bound extract of *S. hystrix* from Gunung Kidul area at dry season showed the highest yield among the other extracts.

There was significant positive correlation ($p < 0.05$, **Table 2**) for linier regression between total phenolic content and % RSA. These results showed that the higher concentration of phenol, the higher binding ability of DPPH. The coefficient of determination (R^2) of total phenolic content and % RSA (**Table 2**) were 0.98 and 0.71 for cytoplasmic and membrand bound extracts, respectively. The R^2 value indicated that scavenging effect of cytoplasmic extracts almost caused by phenolic compound.

Table 1. Analysis of variance of total phenolic content, radical scavenging activity and ferrous ion-chelating ability on various *Sargassum* species from GunungKidul and Jepara coastline

		Total Phenol Content (% PGE)		Radical Scavenging Activity (%)		Ferrous Ion-Chelating Ability (%)	
Source of variation	df	MS	F value	MS	F value	MS	F value
Extract (E)	1	3025.36	15655.10***	4242.19	44375.90***	23842.99	7624.29***
Site (Si)	1	143.46	742.36 ***	1413.04	14781.20***	1858.79	594.39***
Season (Se)	1	81.89	423.78 ***	680.96	7123.25***	388.27	124.16***
E*Si	1	132.31	684.64 ***	51.33	536.96***	1053.29	336.81***
E*Se	1	73.62	380.94 ***	6.86	71.78***	62.15	19.88***
Se*Si	1	0.17	0.89 ns	292.76	3062.48***	192.47	61.55***
E*Si*Se	1	0.69	3.58 ns	37.04	387.48***	180.75	57.80***
Species(E*Se*Si)	14	9.15	47.34 ***	338.82	3544.30***	91.87	29.38***

*** : P<0.0001; ns:non significant

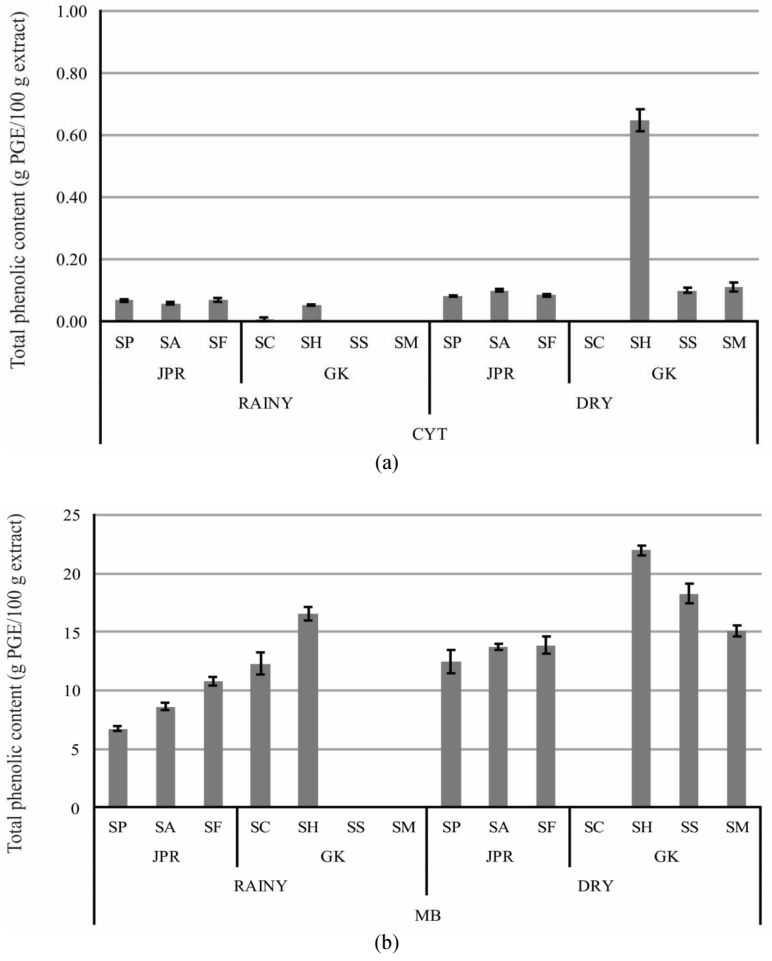
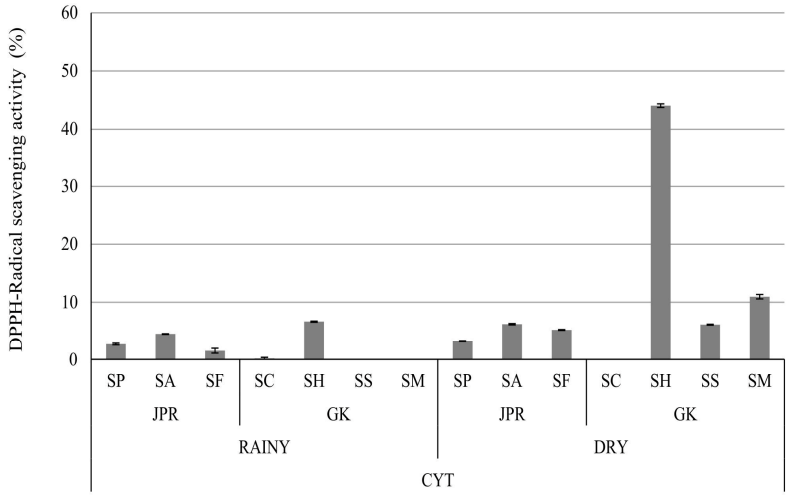
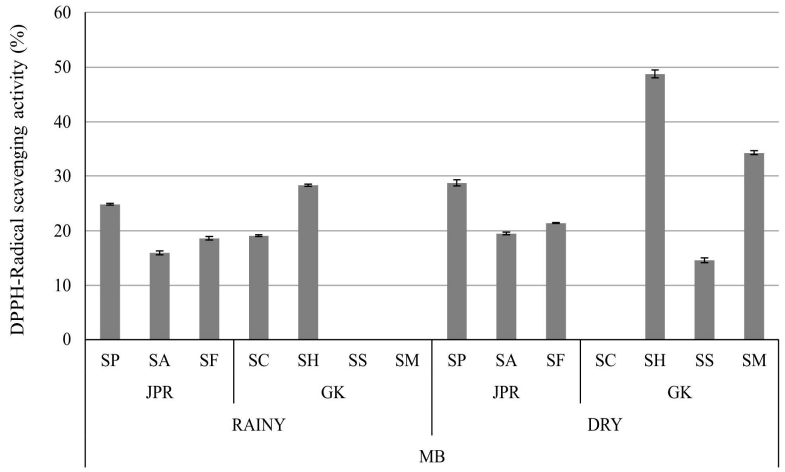


Fig. 1. Total phenolic content (g PGE/100 g extract)) of cytoplasmic (a) and membrane bound (b) extracts on various *Sargassum species* from Gunung Kidul and Jepara coastline. Data were presented as the average of three replicates with error bars indicating Standard Deviation (SD); CYT: cytoplasmic, MB: Membrane Bound, JPR: Jepara; GK: GunungKidul; SP: *S.polyceratium*; SA: *S. angustifolium*; SF: *S. filipendula*, *SC*: *S. cinereum*, SH: *S.hystrix*, *SS*: *S. siliquosum*, *SM*: *S. mcclurei*



(a)



(b)

Fig. 2. DPPH radical scavenging activity (% DPPH-RSA) of 0.45 mg mL^{-1}cytoplasmic (a) and membrane bound (b) extracts on various *Sargassum species* from Gunung Kidul and Jepara coastline. Data were presented as the average of three replicates with error bars indicating Standard Deviation (SD) CYT: Cytoplasmic, MB: Membrane Bound, JPR: Jepara; GK: GunungKidul; SP: *S.polyceratium*; SA: *S. angustifolium*; SF: *S. filipendula*, *SC*: *S. cinereum*, SH: *S.hystrix*, *SS*: *S. siliquosum*, *SM*: *S. mcclurei*

Table 2. Regression, correlation and determination coefficient of cytoplasmic and membrane bound Sargassum extract from GunungKidul and Jepara coastline

Treatments	Fractions	Regression coefficient	Corellation coefficient (r)	Determination coefficient (R^2)
TPCvs RSA	Cytoplasmic	y = 68.19x - 0.15	0.99	0.98
	Membrane bound	y = 1.67x + 1.70	0.84	0.71
TPC vs FIC	Cytoplasmic	y = 15.2x + 4.78	0.59	0.35
	Membrane bound	y = 3.08x + 3.05	0.95	0.90

TPC: Total Phenolic Compound; RSA: Radical Scavenging Activity; FIC: Ferrous ion chelating ability

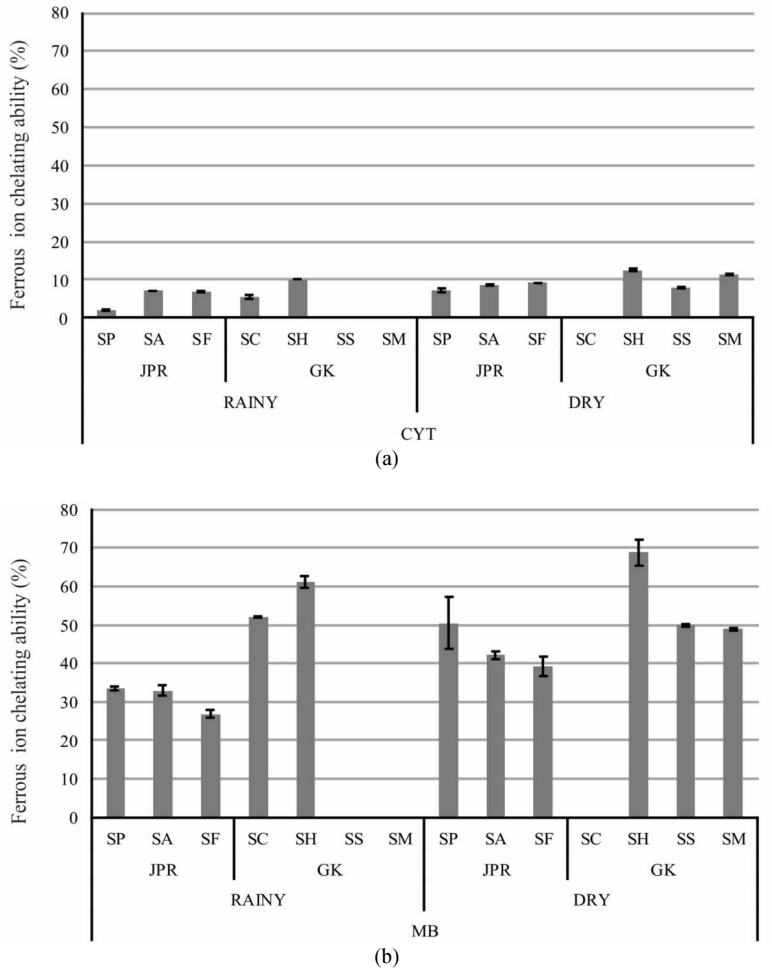


Fig. 3. Ferrous ion-chelating ability (%) of 3.3 mg mL^{-1}cytoplasmic (a) and membrane bound (b) extracts on various *Sargassum species* from Gunung Kidul and Jepara coastline. Data were presented as the average of three replicates with error bars indicating Standard Deviation (SD) CYT: Cytoplasmic, MB: Membrane Bound; JPR: Jepara; GK: Gunung Kidul; SP: *S.polyceratium*; SA: *S. angustifolium*; SF: *S. filipendula*, *SC*: *S. cinereum*, SH: *S.hystrix*, *SS*: *S. siliquosum*, *SM*: *S. mcclurei*

3.3. Ferrous Ion Chelating (FIC) Ability

The ability of cytoplasmic and membrane bound extracts as the ferrous ion-chelating ability were shown in **Fig. 3**. Metal chelating ability of seaweed extracts were tested at concentration of 3.3 mg mL^{-1} for cytoplasmic and membrane bound extracts. The extracts from membrane bound showed higher ferrous ion-chelating activity than cytoplasmic extracts. The activities were in the range of 2.05-12.51% for cytoplasmic and 26.77-68.80% for membrane bound extracts, with the highest activity found in *S. hystrix* (SH) on dry season. Nevertheless, when compared with the positive control 2 ppm EDTA (95.25%), the results of extracts had lower activity. There was significant interaction ($p<0.0001$) on species that nested within (extract x season x site) which means % FIC was influenced by types of extracts, seasons, sites and species (**Table 1 and Fig. 3**).

The ferrous ion chelating ability of cytoplasmic and membrane bound extracts had significant positive correlation with TPC for linier regression ($p<0.05$, **Table 2**). These results showed that the higher concentration of phenol, the higher binding ability to ferrous ion. The coefficient of determination (R^2) between total phenolic content and % FIC (**Table 2**) were 0.35 and 0.90 for cytoplasmic and membrand bound extracts, respectively. The results suggested that

ferrous ion-chelating ability of cytoplasmic extract was not limited to phenolic compounds.

4. DISCUSSION

4.1. Total Phenolic Content (TPC)

The present study investigated that the total phenolic content was influenced by types of extracts, seasons, sites and species. The highest total phenolic content was found at membrane bound extracts of *S. hystrix* from Gunung Kidul at dry season. The result was in accordance with Plouguerne *et al*. (2006) that explained site and season affected the phenolic contents in *Sargassum muticum.* Seasonal variations in brown algae phenolic compounds are species specific, but maximum values are generally observed during the summer and low values during fall and winter (Connan *et al*., 2004). Gunung Kidul coastal area is a rocky shore with longer emersion cycle than Jepara, therefore *Sargassum species* at Gunung Kidul has longer exposed to solar radiation and generally has higher phenolic content. The result was supported by Pavia and Brock (2000) that found the phenolic content in the tissue of *Ascophyllum nodosum*, a kind of brown algae, was regulated by tidal patterns, with greater phenolic concentrations during low tides than during immersion. *Sargassum*, as intertidal species, had photoprotection that represents an efficient physiological adaptation to tolerate deleterious irradiances when low tides coincided with high solar radiation dosages. The temperature in the dry season was higher than in the rainy season, therefore caused a high exposure to solar radiation and also showed the significant effect of Photosynthetically Active Radiation (PAR) on phenolic content (Plouguerne *et al*., 2006). Phlorotannins have been linked to a variety of functions in phaeophytes including protecting algae from high PAR and UV damage (Pavia *et al*., 1997).

The higher phenolic contents at membrane bound extracts showed that phlorotannin may become part of the cell wall and may contribute as defense system. The high level of phlorotannins within outer cortical tissues are well placed for UV interception. Elevated levels of phlorotannins in the tissues are also potentially significant to intercept UV photons before damaging algal cells undergoing UV-vulnerable meiotic or mitotic processes (Ragan and Glombitza, 1986; Tugwell and Branch, 1989; Pavia *et al*., 1997; Swanson and Druehl, 2002).

4.2. Radical Scavenging Activity (RSA)

Recent findings reported that free radical scavenging activity of *Sargassum* extracts were affected by extracts types, seasons, sites and species. The study used DPPH method to test the ability of the antioxidative compounds functioning as proton radical scavengers or hydrogen donors (Singh and Rajini, 2004; Chew *et al*., 2008). DPPH is a compound that possesses a nitrogen free radical and is readily destroyed by a free radical scavenger. The highest % RSA was found at *S. hystrix* from Gunung Kidul at dry season. The extreme condition at Gunung Kidul area encouraged the algae to form a defence system against photodestruction by UV radiation and exhibit radical scavenging properties. The results were supported by Connan *et al*. (2004) that found a positive correlation between phenolic contents and antioxidant activities in brown algae, both in long-term (seasonal) and in short-term (daily) variations. In summer, high solar irradiance could promote the synthesis of radical oxidant substances, thus the increase of phenolic compounds could act as antioxidants. The tropical macroalgae developed an effective antioxidant defence system which might reflect an adaptation to high solar radiation. This screening emphasized the great antioxidant potential, i.e., free-radical, superoxide anion radical scavenging, reducing activity and inhibition of lipid peroxidation (Zubia *et al*., 2007). The photoprotective effect of phenols or phlorotannins depends not only from their accumulation, but also from their high antioxidant and radical-scavenging capacities, which may be involved in other cytoprotective roles (Jimenez-Escrig *et al*., 2001; Lim *et al*., 2002; Connan, *et al*., 2004). In the study, radical scavenging activities of membrane bound extracts were stronger than the scavenging activity of cytoplasmic extract. It was probably caused by the function of the cell walls and membranes as an effective optical barrier attenuating incident UV radiation before reaching intracellular organelles and biomolecules (Holzinger and Lutz, 2006).

There was significant positive correlation between total phenolic compound and radical scavenging activity, indicated that the higher concentration of phenol, the higher binding ability of DPPH. These results were related with *Sargassum* spesies from previous research (Kim *et al*., 2005; Connan *et al*., 2006; Nakai *et al*., 2006; Zubia *et al*., 2008). The R^2 value indicated that scavenging effect of membrane bound (R^2 = 0.71, **Table 2**) was not limited to phenolic compound. Among those isolated from *Sargassum species* were meroterpenoids from S. *siliquastrum* (Jang *et al*., 2005), plastoquinones from *S. micracanthum* (Iwashima *et al*., 2005) and some aromatic compounds from *S. thunbergii* (Seo *et al*., 2007).

4.3. Ferrous Ion Chelating (FIC) Ability

The study showed that FIC abilities were influenced by types of extracts, seasons, sites and species. An

extract with higher binding ability would prevent or inhibit reaction such as a Fenton's type reaction, which generates reactive hydroxyl radicals (Chew *et al*., 2008). The extracts from membrane bound showed higher ferrous ion-chelating activity than cytoplasmic extracts. The higher determination coefficient in membrane bound was caused by phenolic compounds that potent as ferrous ion chelator and could form complexes with metal ions, as protection against toxic metal ions (Ragan and Glombitza 1986; Tooth and Pavia, 2000; Senevirathne *et al*., 2006; Chew *et al*., 2008). The metal chelating ability of polyphenols is related to the number, location of the hydroxyl groups and the presence of ortho-dihydroxy polyphenols, (Khokhar and Apenten, 2003; Santoso *et al*., 2004; Andjelkovic *et al*., 2006). In contrast, the results of cytoplasmic fractions were not as good as membrane bound fractions as metal chelator in agreement with the findings of Saiga *et al*. (2003) and Wang *et al*. (2009) that explained that other components such as polysaccharides, proteins or peptides in the extracts were more effective chelators of ferrous ions than phenolic compounds.

5. CONCLUSION

The study illustrated for the first time that the antioxidant activities of cytoplasmic and membrane bound extract of *Sargassum species* from Gunung Kidul and Jepara coastal, Java Island, Indonesia were influenced by types of extracts, harvest seasons, harvest sites and species. The antioxidant activity of membrane bound extract was higher than cytoplasmic extract. The increasing of DPPH-RSA and FIC were proportional to TPC. The highest antioxidant activity was found at *S. hystrix* from Gunung Kidul area that harvested at dry season.

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