Antioxidant potential of Codium edule, Gracilaria firma and Porphyra crispata from the Philippine coast

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Camille Joy C. Caguicla[1], Angelou V. Alamag[2], Hannah Mae G. Atienza[2], Kezia Margarette C. Bolanos[2], Clarice Faye C. Maralit[2], John Patrick B. Tadiosa[2] and Oliver Shane R. Dumaoal[2]
College of Allied Medical Professions,
Lyceum of the Philippines University, Batangas
camillejoyc1998@yahoo.com[1]

Abstract – *Antioxidants prevent, delay and neutralize the effects of oxidative change and suppression and/or scavenging of free radicals. There is a rapidly growing interest on the discovery of natural sources of antioxidants including those from marine sources such as seaweeds. This study determined the antioxidant activity of different species of seaweeds from the Philippine coast namely Codium edule, Gracilaria firma and Porphyra crispata. Phytochemical contents of the seaweeds revealed the presence of flavonoids and phenolic compounds. The antioxidant potential of the seaweeds was quantitatively assayed using DPPH radical scavenging activity, ferrous ion chelating activity, reducing power activity and TBA-reactive substances at different concentrations (2mg/mL, 3mg/mL, 4mg/ml). Significant differences were observed in DPPH radical scavenging activity, ferrous ion chelating activity, reducing power activity and TBA-reactive substances experiment and the most significant concentration is 4mg/mL in all assays with Gracilaria firma showing the highest antioxidant potential. Further studies regarding other biological activities of the three different seaweeds are recommended.*

Keywords – *antioxidant, phenolic, algae, Gracilaria firma, Codium edule, Porphyra crispata*

INTRODUCTION

Antioxidants are substances that delays, prevents or removes oxidative damage to a target molecule [1]. Free radicals are chemicals that are self-reliant or self-sufficient that possesses one or more unpaired electrons. Free radicals are highly sensitive molecules [2]. The human physique is continuously exposed to free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS). Hydroxyl radical ($OH^{\bullet}$), superoxide radical ($O_2^{\bullet-}$), peroxyl radical ($ROO^{\bullet}$), alkoxyl radical (RO) and nitric oxide ($NO^{\bullet}$) are its examples. Other factors that contribute to formation of free radicals, ROS and RNS include; radiation, cigarette smoke, pollutants and lipid peroxidation. In a healthy human body, the production of ROS and antioxidant enzymes are balanced. Whenever this balance is disturbed over ROS production, thus, oxidative stress will be the result. Oxidative stress contributes to cellular dysfunction that could lead to numerous disorders like Alzheimer's and Parkinson's diseases, cancer, stroke and diabetes [3]. Fortunately, antioxidants are used to protect cells from harm that is brought about by reactive oxygen species or ROS. Antioxidants act as an electron donor that stop reactive oxygen species and free radicals from multiplying that may harm the DNA of the cells that could lead to tumorigenesis or production of tumor [4].

Lately, numerous studies have indicated that the consumption of natural antioxidants has protective effects against the said infections and this protection has been partly ascribed to the presence of several components, such as vitamins, flavonoids, anthocyanins and other phenolic compounds [5]. Over the past few years, there is a rapidly growing interest on the discovery of natural antioxidant; with the main reason that there are evidences which are clinically proven that suggests that consumption of fruits and vegetables reduces the risk of chronic diseases such as cancer. Because of this, considerable works has been done on natural products for the presence of nontoxic antioxidants.

The antioxidant level of seaweeds namely, Porphyra crispata, Gracilaria firma and Codium edule were determined. P.crispata exhibit microscopically dentate blades. The foliose thallus is roundish to reniform or conglobate and monoecious. Gametophytes of P. crispata can be found from late October until early April on the rocky shores when sea temperatures range from 15 to 24 °C and it normally produce monospores [2].

Gracilaria firma was originally described in 1976, based on specimens from Guangdong Province, China, and is currently known from several South-East Asian countries such as Malaysia, Vietnam and the Philippines. This species is characterized by cylindrical axes with a discoid holdfast, branches and branchlets that are constricted at their bases, acute to blunt apices, color

greenish-brown to yellowish-brown, scarce traversing filament between the dense gonimoblast and the pericarp and deep, pot- shaped Verrucosa-type spermatangial conceptacles [6].

Lastly, Codium edule which is locally known as pokpoklo, is a thallus intertwined green algae forming a spongy mass which is a popular edible seaweed sold in the local markets in the Northern Luzon, Philippines [7].

Seaweeds have powerful effects on reducing blood cholesterol and lowering blood pressure, as well as preventing arteriosclerosis [8]. The polysaccharide alginic acid has been demonstrated to have the effect of controlling blood pressure, prevention of constipation and treating various gastro enteric disorders [9]. Therefore, the present study will be undertaken to evaluate the antioxidant activity using free radical scavenging potential, ferrous ion chelating activity, reducing power activity and TBA-reactive substance.

Figure 1. (A) *Codium edule* (B) *Gracilaria firma (C) Porphyra crispata*

MATERIALS AND METHODS

Collection and Identification

The seaweeds were collected in La Union, Ilocos Norte and Calatagan, Batangas. It was identified by Marine Science Institute, University of the Philippines. Fresh seaweeds were washed with distilled water and their hold fasts and epiphytes were removed. The rinsed seaweeds were dried immediately. The seaweed samples were grounded to a powder using a grinder.

Preparation of Extract

Seaweeds were extracted with ethanol as the solvent. In a 1 g of a powdered sample, 10 mL of ethanol were added, and then the mixtures were stirred at room temperature for 24 hours. Each extract was filtered by Whatman no.1 and concentrated using a rotary evaporator at 40˚C for 40 minutes.

Phytochemical screening

This assay was carried out as described by Tiwari, et al [10].

Detection of alkaloids: Extracts were dissolved individually in dilute hydrochloric acid and were filtered using Wagner's test; filtrates were treated with Wagner's reagent (iodine in potassium iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Detection of carbohydrates: Extracts were dissolved individually in 5 ml distilled water and filtered. 1ml of extract were treated with 5 drops of iodine solution, gives blue color indicates the positive test. Detection of saponins: Using Froth test, 0.10 gm of extracts was shaken with 0.5 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Detection of phenols: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenols.

Detection of tannins: To the extracts, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

Detection of flavonoids: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow color indicates the presence of flavonoids.

DPPH radical scavenging activity

This assay was carried out as described by Cho et al., [11] with some modifications. One milliliter of sample (at a concentration of 2mg/ml) was added to 1 mL of 0.2mM DPPH solvent, vortex-mixed, and was allowed to stand at room temperature for 30 mins. Absorbance was measured at 517 nm. The control used was ascorbic acid. The capability of scavenging the DPPH radical was calculated using one minus the absorbance of the extract divided by the absorbance of the control multiplied by 100.

Ferrous ion - chelating activity

This assay was carried out as described by Kumar, et al [12] with some modification. To 1 mL of the extract (at a concentration of 2mg/ml) were added to a solution of 2 mM Ferric chloride (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was then measured at 562 nm. The percentage inhibition is equals to one subtracted from the absorbance of the sample divided by the absorbance of the control multiplied to 100.The control contained ferric chloride and ferrozine, with complex formation molecules.

Reducing power activity

This assay was carried out as described by Farvin and Jacobsen, [13] with some modifications. To 1 ml of extract (at a concentration of 2 mg/ml), 1 ml 0.2 M phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide were added. The mixtures were incubated at 50 °C for 20 min and 1 ml of 10% trichloroacetic acid was added into the reaction mixtures and then centrifuged for 10 minutes at 3000 rpm. The supernatant (2 ml) from the centrifuged mixtures were mixed with 2 ml of distilled water and 0.5 ml of 0.1% ferric chloride in test tubes. After 10 min the solutions were measured at 700 nm using a spectrophotometer. Ascorbic acid was used as a reference as it has good reducing property. Increased absorbance (A700) of the reaction mixture indicated increased reducing power.

TBA-reactive substances (TBARS) experiment

This assay was carried out as described by Cho et al., [11].To 1 *M* potassium phosphate buffer (pH 6.5, 8 mL), 50 uL of Tween-20 and 0.25 ml of tuna oil were added. The mixture was stirred for 10 minutes then KOH and 1:1 HCI was added, and the mixture was adjusted to pH 6.5. To 0.5 mL mixture, 0.5 mL of sample, 0.1 mL of 0.05 *M* FeCl2 solution, and 0.3 mL of distilled water was added. The mixture (1 mL) were then incubated in a water bath at 37°C for 1 hour. To this mixture, 50 uL of 7.2% BHT was added and was mixed well. 2 mL of trichloroacetic acid solution was added then the mixture was heated in the water bath for 15 minutes, was cooled, and was centrifuged for 10 minutes at 2,000 *g*. The absorbance of the resulting solution was measured at 531nm. Distilled water was added as the control instead of the sample extract. Antioxidant capability was calculated based on the absorbance measurements using one subtracted from the absorbance of the extract divided by the absorbance of the control multiplied to 100.

Statistical Analysis

All results were expressed as mean (SEM of three different trials and analyzed with Sigma Stat software. Analysis of Variance (ANOVA) and the Student-Newman-Keuls test were used to assess significant differences ($p< 0.05$) between fractions.

RESULTS AND DISCUSSIONS

Phytochemical analysis

The result of the phytochemical analysis of the extracts revealed that flavonoids and phenols were generally present in all the seaweeds (Table 1).

Table 1. Qualitative phytochemical studies of seaweeds

Study number	Name of compounds	Name of tests	Name of seaweeds		
			Codium edule	*Gracilaria firma*	*Porphyra crispata*
1	Alkaloids	Wagner's test	*present*	*present*	*absent*
2	Carbohydrates	Molisch's test	*absent*	*absent*	*absent*
3	Saponins	Foam test	*present*	*present*	*absent*
4	Phenols	Ferric Chloride test	*present*	*present*	*present*
5	Tannins	Gelatin test	*present*	*present*	*absent*
6	Flavonoids	Alkaline Reagent test	*present*	*present*	*present*

Phenols are known to have antioxidant properties and also have metal chelating properties preventing metals from initiating radical process, They also have anti-microbial, antifungal, anti-atherosclerotic properties and anti-cancer properties [14].. Flavonoids have properties that prevent the oxidative damage of membranes, DNA and proteins thus, preventing diseases to occur. Flavonoid- containing diet may decrease the risk of some cancers to occur and it is also has radical scavenging properties that prevents the oxidative cell damage to occur [15]. Alkaloids, tannins and saponins tested positive for *C. edule* and *G. firma* and tested negative for *P. crispata.* Tannins have anti astringent properties and were used as antiulcer and as antioxidant. Drugs containing tannins were used to treat inflammation, burns and piles [16]- [17].

Saponins are responsible for the antimicrobial property of the seaweeds preventing disease invasion of parasitic fungi. Saponins have anti- inflammatory properties and sold as dietary supplements [15]. Alkaloids are known to have anti herbivore property in plants and it also has antimicrobial property [16]. Carbohydrates tested negative or is absent in all of the three seaweeds. The negativity of carbohydrates may be due to the effects of the processes the seaweeds undergone. The said importance and uses of phytochemicals mentioned above can be used as reference for other studies.

DPPH radical scavenging activity

The DPPH radical scavenging method in this study is used to assess the antioxidant capacity of the seaweed extracts. In comparison to ascorbic acid as the standard antioxidant, the scavenging activity was found to be strongly dependent on concentration, which increases at large concentrations (Table 2).

Table 2. ***Comparison of DPPH Assay against the Positive Control***

Dunnett's multiple comparisons test	Mean Diff.	Significance
Porphyra crispata 4mg/mL	-31.19	<0.0001
Porphyra crispata 3mg/mL	-17.62	<0.0001
Porphyra crispata 2 mg/mL	0.5833	ns
Codium edule 4mg/mL	-20.14	<0.0001
Codium edule 3mg/mL	-1.937	<0.0001
Codium edule 2mg/ml	27.50	<0.0001
Gracilaria firma 4mg/mL	-40.73	<0.0001
Gracilaria firma 3mg/mL	-34.59	<0.0001
Gracilarica firma 2mg/mL	-20.75	<0.0001

All concentrations (2mg/mL, 3 mg/mL, 4mg/mL) of *Gracilaria firma* (69.93%, 83.82%, and 89.89%) exhibited higher scavenging activity than the control (49.18%). On the other hand, *Porphyra crispata* at 3mg/mL (66.80%) and 4mg/ml (80.37%) and *Codium edule at* 3mg/mL (51.14%) and 4mg/mL (69.34%) also exhibited higher scavenging activity than the standard. All extracts aside from *Porphyra crispata* at 2mg/mL (48.60%) and *Codium edule at* 2mg/mL (21.72%) exhibited a relatively high antioxidant activity which was significant. Earlier studies have been validated this observation to point out that seaweeds are potential reservoirs of anti - oxidative compounds [18]. Also, other research revealed that the brown, green and red algae exhibit high radical scavenging activities [19].

Ferrous metal ion chelation

In Table 3, all concentrations (2mg/mL, 3 mg/mL 4mg/mL) of the three seaweeds were significant. Thus, having the same mechanism as the control used which is EDTA. *Gracilaria firma* at concentrations 2, 3 and 4 mg/ml exhibited 80.95%, 80.95% & 71.43% of inhibition, respectively.

Table 3. ***Comparison of Ferrous Ion Chelation against the Positive Control***

Dunnett's multiple comparisons test	**Mean Diff.**	**Significance**
Porphyra crispata 4mg/mL	7.953	<0.001
Porphyra crispata 3mg/mL	15.90	<0.0001
Porphyra crispata 2 mg/mL	33.35	<0.0001
Codium edule 4mg/mL	11.13	<0.0001
Codium edule 3mg/mL	36.53	<0.0001
Codium edule 2mg/ml	52.40	<0.0001
Gracilaria firma 4mg/mL	17.48	<0.0001
Gracilaria firma 3mg/mL	17.48	<0.0001
Gracilarica firma 2mg/mL	25.42	<0.0001

Codium edule, on the other hand, gave 90.48%, 61.90% & 47.62% of inhibition in increasing concentration. Lastly, *Porphyra crispata* exhibited the highest percentage of inhibition of 90.48%, 80.85% & 66.7%. While all of these concentrations led to significant results, the control used still exhibited a greater chelation activity. This reveals that the seaweed extracts are a moderate chelating agent compared to EDTA. Chelators are small molecules that bind very tightly to metal ions. The most readily apparent mechanism by which chelators provide protection is removal of the excess iron from thebody. Once the toxic iron is gone, the body's repair mechanisms can swing into action to correct damage that may have occurred. The antioxidant properties of the natural seaweed extracts can be seen from their potential to chelate transitional metal ions, especially iron and copper. Like the intricated formation between ferrozine, the reagent used, and iron, its color intensity can be decreased by the disturbances through presence of other complexing component. This is attributed to the phenolic compounds from seaweeds like *Codium*, *Gracilaria* and *Porphyra* that consist of dihydroxy groups which can conjugate transition metals, therefore preventing the metal-induced free radical formation [20]. In this assay, we can say that the higher the percentage of inhibition, the higher the anti-oxidant activity of the seaweed.

Reducing power activity

Higher absorbance of the mixture indicates higher reductive potential Jayanthi and Lalitha, [21].

Table 4. ***Comparison of Reducing power activity against the Positive Control***

Dunnett's multiple comparisons test	Mean Diff.	Significance
Porphyra crispata 4mg/mL	-0.0003333	Not Significant
Porphyra crispata 3mg/mL	-0.005667	Not Significant
Porphyra crispata 2 mg/mL	-0.01033	Not Significant
Codium edule 4mg/mL	9.313e-010	Not Significant
Codium edule 3mg/mL	0.004000	Not Significant
Codium edule 2mg/ml	0.007333	Not Significant
Gracilaria firma 4mg/mL	-0.07467	Not Significant
Gracilaria firma 3mg/mL	0.003333	Not Significant
Gracilaria firma 2mg/mL	0.008667	Not Significant

In Table 4, none of the extract except *Gracilaria firma* at a concentration of 4mg/mL exhibited an increase reducing power against ascorbic acid. Data showed that at the concentration of 2mg/mL, 3mg/mL and 4mg/mL, *G. firma* (0.008, 0.013, 0.23) *P. crispata* (0.027, 0.022, 0.017) *Codium edule* (0.01, 0.012, 0.015). The reducing power of extracts increased with the increase of concentration except for *Porphyra crispata.* Same trend has also been reported by Ye et al., [22], Ascorbic acid is known to have a potent antioxidant effect due to the termination of iron ions and donation of electrons that leads to an oxidation-reduction [11]. The solvent used was ethanol that was proved to have a decrease on the activity, an indication that an increase in hydrophilic compounds present in aqueous solution affects the activity. The reducing ability of *Gracilaria firma* was dose dependent (4mg/ml) and significantly higher than the control [23]. Interestingly, the green algae, *Gracilaria firma* exhibited high reducing power, showing that some species extracted in ethanol give a high reducing property [24]. The reducing power is used to transform radical to non-radical compound by donating hydrogen or an electron.

Table 5. ***Comparison of TBARS activity against the Positive Control***

Dunnett's multiple comparisons test	Mean Diff.	Significance
Porphyra crispata 4mg/mL	-9.293	Not Significant
Porphyra crispata 3mg/mL	8.193	Not Significant
Porphyra crispata 2 mg/mL	24.04	<0.0001
Codium edule 4mg/mL	-2.73	Not Significant
Codium edule 3mg/mL	6.01	Not Significant
Codium edule 2mg/ml	29.51	<0.0001
Gracilaria firma 4mg/mL	-8.213	Not Significant
Gracilaria firma 3mg/mL	-0.97	Not Significant
Gracilaria firma 2mg/mL	9.833	<0.05

TBA-reactive substances (TBARS) experiment

In Table 5, the comparison of different concentrations of TBA- reactive sustances is presented. Based from the result, there were decreased antioxidant capability compared to the control, ascorbic acid (69.94%), for 2 mg/mL concentration of *Gracilaria firma* (60.66%), *Codium edule* (40.98%) and *Porphyra crispata* (45.90%). Lesser antioxidant capability has also seen for 3 mg/mL concentration of *Codium edule* (63.93%) and *Porphyra crispata* (62.30%). However, there were increased antioxidant capability for 3 mg/mL and 4 mg/mL of *Gracilaria firma (* 70.49% & 78.69%) , 4 mg/mL concentrations of *Codium edule* (72.13%) and *Porphyra crispata* (78.69%). Antioxidants inhibit lipid oxidation and TBARS assay can measure the percentage inhibition [25]. This means that higher extract concentrations increased the inhibition of lipid oxidation. The concentrations of *Gracilaria firma* (70.49%). In addition, extracts that contained phenolic content were also potent in inhibiting lipid oxidation [25]. Lastly, using ethanol as an extract, exhibited a very high inhibition effect on the formation of TBARS [11]. The TBARS assay is used to measure the potency of an antioxidant in a lipid system.

CONCLUSION AND RECOMMENDATION

Unripe A. bunius extract can reduce silver nanoparticles. The synthesized silver nanoparticles can inhibit MSSA and MRSA, however, their inhibitory activity is lesser than that produced by the standard antibiotics. Therefore, bio-reduced metal nanoparticles using A. bunius can be used as a potential, natural antimicrobial agent against inhibiting Staphylococcus aureus infections.

Future studies on the antimicrobial effects of metal nanoparticles bio-reduced by unripe A. bunius (bignay) fruit extract is highly recommended. Use of other nanoparticles like gold, copper and zinc and various parts like ripe fruits, leaves, bark, and roots of bignay tree are recommended for future testing.

Researchers also recommend increasing the concentration of the synthesized nanoparticles for stronger activity against inhibiting bacteria. Use of other methods in the characterization of the metal nanoparticles like Scanning Electron Microscopy,

Transmission Electron Microscopy, and X-ray Diffraction Assay are likely recommended as well.

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