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PHYTOCHEMICAL SCREENING AND ANTIOXIDANT PROPERTIES OF THE CRUDE EXTRACTS OF PUKPUKLO (*Codium repens)*, BAL-BALULANG *(Hydroclathrus clathratus)* and GAMET (*Porphyra suborbiculata)*

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Abstract

Antioxidants are important components in the body which play a vital role in eradicating free radicals. A number of antioxidants are found in various dietary sources such as seaweeds. Globally, several studies have indicated that seaweeds are rich sources of phenolic compounds and have antioxidant properties. As was found in several studies, seaweeds, when consumed as part of the diet, provide protection against several chronic oxidative stress-related diseases. This study determined the chemical constituents and comparison of the antioxidant properties of selected seaweeds commonly available in Ilocos Norte.

Laboratory experimentation constituted the major portion of the investigation using standard antioxidant drug in measuring the activity of the samples. The experiment focused on the following: seaweeds extraction, phytochemical screening, total phenolics determination using total phenolic content assay and antioxidant property analysis through 2,2 -diphenyl-1-picrylhydrazyl (DPPH) assay, and iron-reducing activity assay.

Results showed that *Codium repens*, *Hydroclathrus clathratus* and *Porphyra suborbiculata* contain carbohydrates, proteins, phytosterols, and phenolics, while only *Hydroclathrus clathratus* had flavonoids. Among the seaweed crude extracts, *P. suborbiculata* possessed the highest total phenolic content. In addition *H. clathratus* manifested the greatest capacity to scavenge-free radicals and *P. suborbiculata* exhibited the strongest iron-reducing power.

Keywords: *antioxidant, phenolics, phytochemical, seaweed*

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Introduction

Non-communicable diseases (NCDs) are already the world's major causes of death, responsible for 36 million deaths in 2008, or 63 percent of the global total, with 78 percent of these deaths occurring in middle- and low-income countries (WHO, 2011). NCDs are the primary causes of death in the Philippines. In 2009, seven of the ten leading causes of deaths are non-communicable in etiology (Ulep, 2012). Thus, lifestyle-related diseases have begun to dominate in the leading causes of death, particularly heart diseases, vascular system diseases, malignant neoplasms, diabetes mellitus, and chronic lower respiratory diseases.

Due to the increasing rate of degenerative diseases caused by cellular degradation and due to the expensive medication, more research studies regarding possible sources of effective drugs, germane and inexpensive in treating degenerative diseases are needed. Through the discovery of new drugs from indigenous plants or marine plants, which are abundant in nature, pharmaceutical companies can provide affordable drugs.

According to Othman, et al (2011), antioxidants have great importance in terms of preventing oxidative stress that may cause several degenerative diseases. Antioxidants are believed to play a very important role in the body defense system against reactive oxygen species (ROS) or free radicals, which are harmful by-products generated during aerobic activity of normal cells. Increasing the intake of dietary antioxidant is believed to assist in keeping antioxidant adequate and the normal physiological function of living system.

Several studies have indicated that seaweeds are a rich source of phenolic compounds and have antioxidant properties (Vinayak, 2011). Seaweed food products provide nutritional benefits, which account for their increasing presence in the Philippine market. Seaweed rolls and salads are popular in the Philippines, as well as in China, Korea, and Japan. Moreover, seaweeds are essential ingredients in some cosmetics, fertilizers, and medicines.

Hence, this study determined and compared the antioxidant properties of selected seaweed species commonly available in Ilocos Norte. These seaweeds are Pukpuklo (*Codium repens*), Bal-balulang (*Hydroclathrus clathratus*), and Gamet (*Porphyra suborbiculata).*

Specifically, it sought to: a) identify constituents of *C. repens, H. clathratus,* and *P. suborbiculata* crude extracts through phytochemical screening; b) compare the *C. repens, H. clathratus,* and *P. suborbiculata* crude extracts in terms of their total phenolic content; c) describe the antioxidant properties of *C. repens, H. clathratus,* and *P. suborbiculata* crude extracts measured through 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and iron-reducing activity assay; d) determine whether a significant difference exists between the mean absorbance of *C. repens, H. clathratus,* and *P. suborbiculata* crude extracts and the reference standard Vitamin C through DPPH Assay and iron-reducing activity assay, and e) identify the half-inhibition concentration (IC_{50}) values of the *C. repens, H. clathratus,* and *P. suborbiculata* crude extracts in DPPH assay and iron-reducing activity assay.

Methodology

Seaweeds Collection, Preparation and Extraction

Three types of seaweeds, locally known as Pukpuklo, Bal-balulang and Gamet, were obtained from the seaweed bank of the Crops Research Laboratory of the Mariano Marcos State University. The seaweed samples were subjected to air

drying, cut into small pieces, and ground into fine particles using a grinder, kept in an air tight container, and properly stored in a refrigerator. The extraction process was done following the Ismail and Hong's method (2002). The ground sample was weighed and transferred into an Erlenmeyer flask. Methanol was added and stirred for one hour with a magnetic stirrer. The extraction mixture was left to sediment for at least 24 hours before the extract was separated from the residue by filtration using Whatman filter paper #2. The methanolic extract solvent was removed under reduced pressure at 40° C using a rotary evaporator. Seaweed crude extracts were produced in triplicates.

Phytochemical Screening

Qualitative phytochemical analyses were done following the procedures of Kaur, et al. (2011) and Himesh, et al (2011).

Total Phenolic Content (TPC) Assay

The TPC was determined by spectrophotometry using gallic acid as standard, according to the method described by the International Organization for Standardization (ISO) 14502-1. A 0.5mL of the diluted seaweed crude extract was transferred in triplicate to separate tubes containing 2.5mL of a 1/10 dilution of Folin-Ciocalteu's reagent in distilled water. Then 2mL of sodium carbonate solution (7.5% w/v) was added. The tubes were then allowed to stand at room temperature for 60min before absorbance at 765nm was measured against blank reagent. The TPC was expressed as gallic acid equivalents (GAE) in microgram/ mg extract. The concentration of phenols in samples was derived from a standard curve of gallic acid ranging from 0.05 to 0.250mg/ ml (Pearson's correlation coefficient: r^2 =0.9626).

Measurement of Free Radical Scavenging Property using DPPH Assay

Antioxidant property was measured from the modified method of Bozin, et al (2006) and Nyaa, et al (2009) wherein different dilution extracts (25 mcg/ml – 150 mcg/ml) amounting to 1 mL were mixed with 2mL of 30uM DPPH solution. After a 30 minute-incubation period at 37°C, the absorbance was recorded at 517nm. Ascorbic acid was used as a positive control for this assay. The antioxidant activity is expressed as:

$$\text{Inhibition (\%)} = \frac{(\text{Abscontrol} - \text{Abssample})}{\text{Abscontrol}} \times 100\%$$

Where:

$A_{control}$ = Absorbance reading of the control
A_{sample} = Absorbance reading of the sample

Half maximal-inhibitory concentration (IC_{50}), the amount of sample necessary to decrease the initial DPPH concentration by 50%, was determined from the plotted graph of the percentage of radical scavenging activity (% inhibition) versus the extracts concentration [(Norshazila, et al (2010)]. The IC_{50} values were specifically determined using linear regression analysis.

Iron-Reducing Activity Assay

The iron-reducing assay was modified and derived from the method of Rana, et al (2010). The reaction mixture containing 1mL ortho-phenanthroline, 2mL ferric chloride, and 2mL seaweed crude extracts at various concentrations ranging from 25 to 150µg/mL in a final volume of 5mL was incubated for 10min at 37°C. The absorbance was recorded at 510nm. Ascorbic acid was added and absorbance obtained was taken as equivalent to 100% reduction of all ferric ions. Blank was

$$\text{Reduction power (\%)} = 1-\left(1-\frac{A_s}{A_c}\right) \times 100$$

Where:

A_C = absorbance of standard at maximum concentration tested
A_S = absorbance of sample

carried out without drug. Experiment was performed in triplicate.

Half maximal inhibitory concentration (IC_{50}), the amount of sample necessary to decrease the initial DPPH concentration by 50%, was determined from the plotted graph of the percentage of radical scavenging activity (% inhibition) versus the extracts concentration (Norshazila, et al 2010). The IC_{50} values were specifically determined using the linear regression analysis.

Statistical Treatment

To show whether *C. repens, H. clathratus,* and *P. suborbiculata w*ere comparable with the reference standard in different concentrations as to absorbance readings through DPPH assay and iron-reducing assay using spectrophotometer, the researcher employed one-way Analysis of Variance and further analyzed through Least Significant Difference. All experiments were performed in triplicates.

Results and Discussion

Phytochemical Constituents of Seaweeds

As shown in Table 1, *C. repens, H. clathratus and P. suborbiculata* yielded positive results for the primary metabolite carbohydrates and proteins as confirmed by the Molisch's Test and Xanthoproteic test where typical nutritional analyses of seaweeds have identified high levels of carbohydrates and proteins (MacArtain, et al, 2007).

Likewise, phytosterols and phenolics were present on all seaweed crude extracts as confirmed by Liebermann Burchard's test and Ferric chloride test. Flavonoids were present in the extract of *H. clathratus* as manifested by the positive result in the lead acetate test while *C. repens* and *P. suborbiculata* obtained a negative result.

The presence of phytochemicals suggests that the seaweeds possess antioxidant activity. For the phenolics, the antioxidant properties are mainly due to their reduction - oxidation properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers (Guendez, et al, 2005). It should be noted that flavonoids possess antioxidant properties *in vitro* and *in vivo* and contain a number of phenolic hydroxyl groups attached to ring structures, which confer the antioxidant activity (Kong, et al 2003).

Total Phenolic Content

The basic mechanism of the Folin-Ciocalteau assay is an oxidation/reduction reaction based on the reduction oxidation properties of antioxidant compounds that can react with the Folin-Ciocalteau reagent enhancing the measurement of phenolic concentration (Norshazila, 2010).

Results of TPC assay, as shown in Table 2, were expressed as gallic acid equivalent (GAE) in microgram/milligram seaweed extract. *P. suborbiculata* had the highest total phenolic content of 21.520µg GAE/mg followed by *C. repens and H. clathratus* with 4.638 and 3.587µg GAE/mg, respectively.

Analysis using LSD revealed that *P. suborbiculata* exhibited the highest total phenolic content. Due to the presence of phenolic compounds, seaweeds suggest to have multiple biological activities such as antimutagenicity, anticarcinogenicity, anti-aging, and antioxidant (Kosem, et al 2007).

Table 1. Phytochemical constituents of seaweed crude extracts

TEST FOR CONSTITUENT	CONSTITUENT DETECTED	SEAWEED CRUDE EXTRACT		
		Codium repens	*Hydroclathrus clathratus*	*Porphyra suborbiculata*
I. Detection of Alkaloids				
• Mayer's test	**Alkaloids**	-	-	-
• Dragendorff's test	**Alkaloids**	-	-	-
• Hager's test	**Alkaloids**	-	-	-
• Wagner's test	**Alkaloids**	-	-	-
II. Detection of Carbohydrates				
• Molisch's test	**Carbohydrates**	+	+	+
• Fehling's test	**Reducing sugar**	-	-	-
III. Detection of Glycosides				
• Modified Borntrager's test	**Anthraquinone**	-	-	-
• Legal's test	**Cardiac glycoside**	-	-	-
IV. Detection of Saponins				
• Froth test	**Saponins**	-	-	-
V. Detection of Phytosterol				
• Cupric acetate test	**Diterpenes**	-	-	-
• Salkowski's test	**Triterpenes**	-	-	-
• Liebermann Burchard's test	**Phytosterols**	+	+	+
VI. Detection of Phenolic compounds				
• Ferric chloride test	**Phenolics**	+	+	+
• Gelatin test	**Tannins**	-	-	-
VII. Detection of flavonoids				
• Alkaline reagent test	**Flavonoids**	-	-	-
• Lead acetate test	**Flavonoids**	-	+	-
VIII. Detection of Proteins				
• Xanthoproteic test	**Proteins**	+	+	+

*(-); (+) presence

Table 2. Total phenolic content of seaweed crude extracts

SAMPLE	MEAN ABSORBANCE	TOTAL PHENOLIC CONTENT (µg GAE/mg extract)
Codium repens	0.025 [b]	4.638
Hydroclathrus clathratus	0.019 [c]	3.587
Porphyra suborbiculata	0.116 [a]	21.520

[abc] *Different superscripts within column indicate significant difference at p<0.05.*

Table 3. Free radical scavenging activity of the seaweed crude extracts

CONCENTRATION	SAMPLE	MEAN ABSORBANCE	% FREE RADICAL INHIBITION
25µg/mL	*Porphyra suborbiculata*	0.697[b]	34.307
	Codium repens	0.697[b]	34.307
	Hydroclathrus clathratus	1.020[c]	3.864
	Ascorbic acid	0.148[a]	86.082
50µg/mL	*Porphyra suborbiculata*	0.693[d]	34.653
	Codium repens	0.675[b]	36.412
	Hydroclathrus clathratus	0.688[c]	35.187
	Ascorbic acid	0.095[a]	91.078
100µg/mL	*Porphyra suborbiculata*	0.658[d]	38.014
	Codium repens	0.606[b]	42.884
	Hydroclathrus clathratus	0.651[c]	38.611
	Ascorbic acid	0.079[a]	92.554
150µg/mL	*Porphyra suborbiculata*	0.654[d]	38.360
	Codium repens	0.499[c]	52.969
	Hydroclathrus clathratus	0.480[b]	54.791
	Ascorbic acid	0.065[a]	93.842

[abcd] *Different superscripts within a column per level of concentration indicate significant differences and same letters indicate no significant difference at $p < 0.05$.*

DPPH Assay

Primary antioxidant property of the seaweeds was measured through DPPH assay, because it is one of the most effective methods for actively evaluating the concentration of radical-scavenging materials actively by a chain-breaking mechanism. The reduction capability of DPPH is determined by the decrease in its absorbance at 517nm induced by antioxidants (Norshazila, et al, 2010). Antioxidant can be categorized into two main types called primary and secondary; each type is responsible for different mechanisms (Lim, *et al*, 2007). DPPH assay reaction depends on the ability of the samples to scavenge free radicals, which is visually noticeable as the color changes from purple to yellow due to hydrogen-donating ability (Ajila, *et al*, 2007).

In terms of the level of concentration of 150µg/mL among seaweeds crude extracts, *H. clathratus* had the highest percentage of free radical inhibition followed by *C. repens* and *P. suborbiculata* (Table 3). However, the lowest value was manifested by *H. clathratus* in the 25µg/mL level of concentration, while *P. suborbiculata* and *C. repens* had the same percentage of free radical inhibition. In 50µg/mL, *H. clathratus* was the highest followed by *C. repens* and *P. suborbiculata* while in 100 µg/mL *C. repens* reached the highest free-radical inhibition percentage compared with the two seaweed crude extracts.

It can be deduced from the data that *P. suborbiculata* slightly increased the free radical inhibition as affected by the concentration, while *C. repens* and *H. clathratus* showed notable increase. The

scavenging activity of all samples on the DPPH radical was found to be strongly dependent on concentration. Thus, it can be noted that the lower the mean absorbance is, the greater the primary antioxidant activity or the free radical-scavenging activity takes place (Siddhuraju, *et al*, 2002).

The ANOVA results revealed that there are significant differences ($p<0.05$) among the mean absorbance levels among all concentrations ($p<0.05$). Based on further analysis using LSD, the standard reference, Ascorbic acid, exhibited stronger antioxidant activity as manifested by a very high percentage on free-radical inhibition compared to the crude extracts of the three seaweeds. But among the seaweeds crude extracts, *H. clathratus* had the highest free radical-scavenging activity.

Iron-Reducing Activity Assay

Secondary antioxidant activities of the extracts were measured using iron-reducing activity assay. It was reported that reducing agents, which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion (Norshazila, 2010). O-phenanthroline quantitatively forms complexes with Fe+2, which get disrupted in the presence of reducing agents. The crude extract may interfere with the formation of a ferrous-o-phenanthroline complex, thereby it may suggest that the extract has reducing activity. Additionally, absorbance obtained was taken as equivalent to 100% reduction of all ferric ions (Rana, et al, 2010). *O*-phenanthroline is selective agent for ferrous ion. It is used for determining the extent of ferric ions reduction to ferrous ions by antioxidants (Qureshi, et al, 2010).

As shown in Table 4, there were no large differences among seaweed extracts in their iron-reducing properties. The highest iron-reducing effect among the seaweed extracts as manifested by their mean absorbance was shown by *P. suborbiculata* that was followed by *H. clathratus* and *C. repens* in the 100mcg/mL and 150mcg/mL concentration. However, in the concentration of 25mcg/mL and 50mcg/mL, reducing effect of *P. suborbiculata* was lower than *H. clathratus.* Thus, the higher the absorbance is, the greater the possibility of secondary antioxidant activity to occur (Qureshi, et al, 2010).

Furthermore, ANOVA results showed that there were significant differences ($p<0.05$) among the mean absorbance levels across all concentrations. All seaweed crude extracts showed lower iron-reducing effect as compared with the standard Ascorbic acid, which obtained the highest iron-reducing activity as further analyzed using LSD. However, *P. suborbiculata* had the highest iron-reducing effect compared with the other seaweed crude extracts.

Half Inhibition Concentrations (IC_{50})

Table 5 shows the specific IC_{50} values of the seaweed crude extracts. *H. clathratus* had the highest capacity to scavenge free radicals with 130.30µg/mL concentration and *P. suborbiculata* had the highest iron-reducing effect with 324.12µg/mL concentration. Thus, a lower IC_{50} value suggests better radical scavenging activity and better iron-reducing power (Marijana, et al, 2012).

Relationships among the Total Phenolic Content (TPC), DPPH Assay and Iron-Reducing Activity Assay

Based on the results, a negative relationship between the TPC and DPPH assay exists. TPC assay provides only a crude estimate on the total phenolic components of the seaweed extracts, whereby the DPPH assay is not specific to polyphenols but to many interfering compounds that may react with the reagent,

Folin-Ciocalteu. The responses of phenolics for antioxidant activity estimated by various methods also depend on their chemical structures (Zhao, *et al*, 2007). Furthermore, Tawaha, *et al* (2007) suggest that the negative correlation between TPC and antioxidant activity could be attributed to the TPC that does not necessarily incorporate all the antioxidants present in an extract such as betalain containing both phenolic and non-phenolic structures. Similarly, various phenolic compounds respond differently to the DPPH assay, depending on the number of phenolic groups they have. This explains why *P. suborbiculata* had the highest TPC and least in the DPPH assay, while *H. clathratus* had the highest percentage on the free-scavenging activity and the least TPC.

The results further suggest that not all compounds in the seaweeds extracts, which could scavenge free radicals have favorable iron-reducing effects. The iron-reducing activity of seaweed extracts could partially depend on the functional groups and the contents of individual functional groups in seaweed extracts (Zhao, et al, 2007). Therefore, antioxidant activity of the

Table 4. Iron reducing activity of the seaweed crude extracts

CONCENTRATION	SAMPLE	MEAN ABSORBANCE	% REDUCING EFFECT
25mcg/ml	*Codium repens*	0.022^{d}	5.6
	Hydroclathrus clathratus	0.038^{b}	9.6
	Porphyra suborbiculata	0.026^{c}	6.6
	Ascorbic acid	0.322^{a}	81.5
50mcg/ml	*Codium repens*	0.024^{d}	6.1
	Hydroclathrus clathratus	0.049^{b}	12.4
	Porphyra suborbiculata	0.028^{c}	7.1
	Ascorbic acid	0.343^{a}	86.8
100mcg/ml	*Codium repens*	0.032^{d}	8.1
	Hydroclathrus clathratus	0.054^{c}	13.7
	Porphyra suborbiculata	0.073^{b}	18.5
	Ascorbic acid	0.372^{a}	94.2
150mcg/ml	*Codium repens*	0.040^{d}	10.1
	Hydroclathrus clathratus	0.074^{c}	18.7
	Porphyra suborbiculata	0.093^{b}	23.5
	Ascorbic acid	0.395^{a}	100

^{Abcd} *Different superscripts within a column per level of concentration indicate significant differences and same letters indicate no significant difference at $p < 0.05$.*

Table 5. IC_{50} (ug/ml) values of the seaweed crude extracts

SEAWEED	DPPH ASSAY	IRON-REDUCING ASSAY
Codium repens	137.16	1,232.13
Hydroclathrus clathratus	130.30	626.90
Porphyra suborbiculata	454.76	324.12

seaweeds extracts could not be predicted based on their TPC only. This is due to the synergism of polyphenolic compounds with one another or with other components present in an extract that may contribute to the overall observed antioxidant activity (Ordonez, et al, 2005).

Conclusions and Recommendations

Anchored on the findings of the study, C. *repens*, *H. clathratus,* and *P. suborbiculata* contain carbohydrates, proteins, phytosterols, and phenolics, while only *H. clathratus* has flavonoids.

Among the seaweeds crude extracts, *P. suborbiculata* exhibits the highest total phenolic content and the greatest ability to suppress the formation of free radicals as measured in the iron-reducing assay, while *H. clathratus* manifests the greatest capacity to scavenge free radicals in the DPPH assay. Thus, seaweeds are possible sources of antioxidants that help, protecting the body from degenerative diseases.

Nonetheless, in order to gain more comprehensive insights on the seaweed antioxidant activities, further studies on purification, identification, and quantification of each phenolic compound and other nonphenolic compounds are indispensable.

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