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Bioactive properties and therapeutic potential of *Padina australis* Hauck (Dictyotaceae, Ochrophyta)

Arguelles, E. D. L. R. [1*] **and Sapin, A. B.**[2]

[1]Philippine National Collection of Microorganisms, National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippine Los Baños, College, Laguna, 4031, Philippines; [2]Food Laboratory, National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños, 4031, Philippines.

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Abstract Seaweeds are known sources of biologically active substances with diverse bioactive properties important in the synthesis of medically important novel drugs. The bioactive properties of brown macroalga, *Padina australis* Hauck were studied. Results showed that the seaweed contain a total phenolic content of 13.85 ± 0.04 mg GAE/g. Antioxidant efficiency of *P. australis* are characterized by having potent $ABTS^+$ scavenging activity and high copper reduction capacity with IC_{50} value of 138 μg/ml and 24.47 μg/ml respectively. Evaluation of tyrosinase and elastase inhibition properties showed that *P. australis* extract has potent inhibitory activity with IC_{50} of 32 μg/ml and IC_{50} of 93 μg/ml, respectively more effective than kojic acid and tocopherol. In addition, *in vitro* assessment of alpha-glucosidase and alpha-amylase inhibition property of the alga showed that *P. australis* extract have effective inhibitory activity with IC_{50} values of 5.90 μg/ml and 41 μg/ml, respectively, more potent as compared to acarbose (standard anti-diabetic drug). The seaweed extract exhibited potent antibacterial activities against medically important bacterial pathogens such as *Klebsiella pneumoniae* (Minimum Inhibitory Concentration (MIC) = 125 μg/ml), Methicillin-resistant *Staphylococcus aureus* (MIC = 250 μg/ml), *Pseudomonas aeruginosa* (MIC = 125 μg/ml), and *Staphylococcus aureus* (MIC = 250 μg/ml). The current investigation is a pioneering study in the Philippines that shows the potential of *P. australis* as source of bioactive compounds with important pharmacological applications.

Keywords: Biological activity; Chemical composition; Marine; Philippines; Seaweeds

Introduction

In the last century, seaweeds have drawn pharmaceutical scientists' attention as several species of this organism possess or synthesize natural compounds with a vast array of biological activities (Sari *et al.,* 2019; Lee *et al.,* 2020). These bioactive compounds are generated by these organisms as an adaptive strategy against oxidizing substances that affect the macroalgae

[*] **Corresponding Author**: Arguelles, E. D. L. R.; **Email:** edarguelles@up.edu.ph

because of their exposure to harsh environmental conditions in the marine habitat (Mekinić *et al.*, 2019; Arguelles and Sapin, 2021a,b; Arguelles, 2021b; Mekinić *et al.*, 2021). Seaweeds are heterogeneous photosynthetic group of organisms and are divided into three main groups (red, brown, and green algae). Among these groups of algae, brown seaweeds are considered as the largest class and are likely the most chemically diverse, as these macroalgae possess unique kinds of bioactive compounds such as polyphenols. Phenolic compounds (like phloroglucinol, bromophenols, and phlorotannins) from brown seaweeds serve a critical role in protecting the alga against pathogenic bacteria and other marine herbivores. These compounds are also involved in seaweeds protection to counter oxidative damage, as these compounds posess potent antioxidant activity. In addition, biological properties like antidiabetic, anti-inflammatory, anti-allergic, anti-angiogenic, anticoagulant, tyrosinase and elastase inhibition effects were also documented from brown seaweeds making it as the most widely studied group of seaweeds (Mekinić *et al.*, 2019; Arguelles and Sapin, 2021a; Arguelles, 2021b; Mekinić *et al.*, 2021). Other bioactive substances such as peptides, minerals, polysaccharides, polyunsaturated fatty acids, sterols, and pigments (carotenoids) can also be found from brown seaweeds which can also contribute to the potent biological activities of this organism (Sari *et al.*, 2019; Lee *et al.*, 2020).

The brown alga *Padina australis* Hauck is an algal species that is affiliated to the family Dictyotaceae, order Dictyotales, and class Phaeophyceae. Among the different species from the genus *Padina*, *P. australis* is considered as one of the most widespread species that dwells in the coastal zones of temperate and tropical marine areas worldwide (Čagalj *et al.*, 2021). The vast distribution of this species makes it a suitable and interesting choice for marine natural products research, especially considering that the Philippine strains of this brown macroalgae have been considered as an underutilized marine resources with rich and diverse biological activities (Lee *et al.*, 2020; Arguelles, 2021b; Čagalj *et al.*, 2021). The Philippine marine ecosystem is known to have several seaweed species with diverse bioactive properties yet to be explored. However, only a few studies were documented about the nutritional properties, antibacterial and antioxidant activities of these organisms. Previously, several species of brown algae (*Sargassum ilicifolium*, *Sargassum aquifolium*, *Sargassum vulgare*, *Sargassum siliquosum*, *Turbinaria ornata*, *Padina australis*, *Turbinaria decurrens*, and *Codium intricatum*) were reported to have diverse biological activities (Canoy and Bitacura, 2018; Arguelles *et al.*, 2019; Arguelles, 2020; Arguelles and Sapin, 2020a,b,c; Arguelles and Sapin, 2021b; Arguelles, 2021b) but only two properties (cytotoxicity and antiangiogenic activity) were reported specifically for *Padina* *australis*. Thus, the current study aims to study for the first time in the Philippines other bioactive properties of *Padina australis* with potential use for the development of novel compounds for pharmaceutical application. The study was conducted to know the total phenolic content (TPC), antioxidant (using $ABTS^+$ and copper reduction antioxidant capacity (CUPRAC) assay), antibacterial, antidiabetic as well as tyrosinase and elastase inhibition activities of *P. australis*. In addition, correlation analysis on the phenolic content of the seaweed extract and its antioxidant activity was established.

Materials and methods

Seaweed sampling and collection

Padina australis was collected on 07 March 2021 during low tide condition in the coast of General Nakar (Lat. 14° 47’ 36.66” N; Long. 121° 37’ 25.01” E), Quezon, Philippines. The algal biomass was gently scrubbed, to remove epiphytes, excess sand particles, and other necrotic parts of the algal sample. It was then repeatedly rinsed with sterile distilled water prior to oven-drying at 60 ℃ for 12 hours. The dried seaweed sample was pulverized (250–500 μm) before subjecting it for solvent extraction. The taxonomic identification was done using taxonomic keys of Trono (1997) as well as Algae Base (web site: www.algaebase.org) and was verified by the algae curator of the National Institute of Molecular Biology and Biotechnology (BIOTECH), Laguna, Philippines.

Preparation of seaweed extract

Biomass of *P. australis* (1 gram) was subjected to solvent extraction following the protocol of Gao *et al.*, (2002). The pulverized algal biomass was extracted using 30 ml acidified methanol (1 HCl: 80 CH_3OH: 10 H_2O) in an ultrasonic bath for 30 minutes with continuous stirring for 1 hour. The sample mixture was then centrifuged at 12,000 rpm for 20 minutes at a temperature of 20^{o}C. The algal extract was further concentrated using a rotary evaporator (BUCHI Rotavapor®) set at 40 ℃ under reduced pressure. The concentrated seaweed extract was kept under refrigerated condition (4 ℃) to preserve its biological activity for use to different biological assays needed in the study (Arguelles and Sapin, 2020a).

Determination of total phenolic content (TPC)

The TPC of *P. australis* was analyzed using Folin-Ciocalteu assay (Nuñez-Selles *et al.*, 2002). It is presented as microgram of gallic acid equivalent (GAE) per gram of the seaweed extract (calibration curve equation: y = 0.006415x – 0.0140, R^2 = 0.99978). Initially, about 0.5 ml of *P. australis* crude extract was mixed with 0.5 ml of Folin-Ciocalteau's reagent and 0.5 ml 10% sodium carbonate solution for 1 min. The mixture was thoroughly mixed and was set-aside at room temperature for 5 min. The volume of the reaction mixture was further adjusted using 5 ml sterile distilled water. The absorbance reading of the sample mixtures and control were taken using an Ultraviolet-Visible spectrophotometer (Shimadzu, Kyoto, Japan) at 720 nm wavelength.

Antioxidant activities

The antioxidant activities of *P. australis* extract were assessed using $ABTS^+$ radical scavenging assay and CUPRAC assay. Two different antioxidant assays were used in this study to show the different mechanisms responsible in the antioxidant activities of *P. australis* extract.

$ABTS^+$ (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) scavenging assay

$ABTS^+$ scavenging assay is a simple antioxidant activity assay wherein $ABTS^+$ is transformed to its radical cation by the addition of sodium persulfate (blue in color) and absorbs light at 734 nm. This radical cation is very reactive to several compounds (such as phenolic compounds) and upon reaction converts the radical cation (blue in color) to its neutral (colorless) form (Arguelles and Sapin, 2020a).

$ABTS^+$ scavenging assay for *P. australis* was done following the methods of Re *et al.*, (1999) with few modifications in the procedure. Briefly, 40 μl of *P. australis* extract prepared in different concentrations (35.0 – 175.0 μg GAE/ml) and 40 μl of 90% methanol (for the control) were mixed with 3 ml of $ABTS^+$ radical mixture with an initial absorbance reading of 0.72 ± 0.05 at 734 nm. The reaction sample mixtures were stirred thoroughly and kept at ambient temperature for 5 min. Absorbance readings of each prepared reaction sample solutions were noted at 734 nm and the $ABTS^+$ inhibition (%) was calculated using the equation:

$$ABTS^+ \text{ Inhibition (\%)} = \frac{Abs_{734}\text{ (control)} - Abs_{734}\text{ (sample)}}{Abs_{734}\text{ (control)}} \times 100$$

where A_{sample} is the absorbance reading of the sample (algal extract) and $A_{control}$ is the absorbance reading of the control (ascorbic acid). The $ABTS^+$

inhibition activity (%) was plotted with different prepared concentrations of *P. australis* extract. IC_{50} of the seaweed extract was noted as the concentration the extract that exhibited 50% $ABTS^+$ radical scavenging activity.

Copper reduction antioxidant capacity (CUPRAC) assay

The copper reduction antioxidant capacity assay was done following the procedure of Alpinar *et al.*, (2009). This method measures the capacity of *P. australis* extract to reduce (Cu (II)-Neocurpine) to colored Cu (I)- Neocurpine chelate, which shows a maximum absorbance at 450 nm. Briefly, 1 ml each of 0.01 M $CuCl_2$ solution, 1 M ammonium acetate buffer (pH 7) and 0.0075 M neocuproine were mixed in sterile test tubes containing 0.5 ml of *P. australis* extract (5.0, 10.0, 15.0, 20.0 and 25.0 µg GAE/ml) and ascorbic acid (standard antioxidant) (Arguelles *et al.* 2021a). The total volume for each reaction sample mixtures were adjusted to 4.1 ml using a sterile distilled water and were kept at ambient temperature for 30 min. The absorbance reading for both the *P. australis* extract and ascorbic acid concentrations against a reagent blank was noted at 450 nm (Arguelles, 2018).

Tyrosinase inhibition assay

The whitening property of *P. australis* extract was evaluated *in vitro* via tyrosinase inhibition assay using the protocol of Hapsari *et al.*, (2012) with slight modifications. Initially, chemical solutions of 5mM DOPA (3,4-dihydroxy-L-phenylalanine, Sigma D-9628), mushroom tyrosinase (250 units/ml, Sigma T- 3824), and 0.1M potassium phosphate buffer, pH 6.5 were prepared. An aliquot of 40 µl DOPA is mixed with 40 µl of *P. australis* extract (at varying concentration: 15.0, 30.0, 45.0, 60.0, and 75.0 µg GAE/ml) or 40 µl buffer (for the control) in a 96-well microtiter plate. The total volume of each reaction sample mixture was adjusted to 160 µl by adding 40 µl of phosphate buffer and mushroom tyrosinase. The microtiter plate containing the reaction sample mixtures was kept for 15 min at ambient room temperature. The absorbance reading was taken using a microtiter plate reader at 490 nm wavelength. Percent tyrosinase inhibition was calculated using the equation below:

$$\text{Inhibition (\%)} = \left(\frac{A_{\text{control}} - (A_{\text{sample}} - A_{\text{blank}})}{A_{\text{control}}}\right) \times 100$$

where A_{sample} is the absorbance reading of the sample (seaweed extract), A_{blank} is the absorbance reading of the blank, and $A_{control}$ is the absorbance reading of the control. Kojic acid was used as the positive control in the assay.

Elastase inhibition assay

The anti-aging and anti-wrinkling property of *P. australis* extract was evaluated using elastase inhibition activity assay following the procedure of Moon *et al.* (2010). Initially, solutions of N-succinyl-$(ALA)_3$-p-nitroanilide (25 mM, Sigma S-4760), elastase from porcine pancreas (50 ug/ml, Sigma E-7885) and 0.2M TRIS-HCl buffer, pH 8.0 were prepared. An aliquot (40 µl) of the *P. australis* extract or 40 µl buffer (for the control) were thoroughly mixed with 40 µl N-succinyl-$(ALA)_3$-p-nitroanilide in clean sterile test tubes. The volume of the reaction mixture was further adjusted to 1 ml using phosphate buffer and 40 µl elastase was added last in the solution. On the other hand, the blank tube was the one without the enzyme solution. After 20 min of incubation, 2 ml of TRIS-HCl buffer were added in the reaction mixtures and the absorbance reading of each sample was measured at 410 nm wavelength. The percent elastase inhibition was calculated using the equation:

$$\text{Inhibition (\%)} = \left(\frac{A_{\text{control}} - \left(A_{\text{sample}} - A_{\text{blank}}\right)}{A_{\text{control}}}\right) \times 100$$

where A_{sample} is the absorbance reading of the sample (algal extract), $A_{control}$ is the absorbance reading of the control and A_{blank} is the absorbance reading of the blank. Tocopherol was used as the positive control in the assay.

Antidiabetic activities

The antidiabetic properties of *P. australis* extract were assessed using α-glucosidase and α-amylase inhibition assay. Two different antidiabetic assays were used in this study to show the potential of *P. australis* extract in suppressing key carbohydrate hydrolyzing enzymes such as α- glucosidase and α-amylase.

α-glucosidase inhibition assay

The potential of *P. australis* extract to inhibit α-glucosidase was evaluated following the procedure of Nair *et al.*, (2013) via spectrophotometric assay using *p*-nitrophenyl-α-glucopyranoside (*p*NPG) as a substrate. In this assay, the occurrence of α-glucosidases in algal extract converts *p*NPG (substrate) to *p*-nitrophenol (*p*NP) and is measured spectrophotometrically at a wavelength of 410 nm. Initially, a mixture containing 75 µl of α – glucosidase (2.5 U/ml), 100 µl of *P. australis* extract or 100 µl of 0.1 M phosphate buffer pH 6.8 (for the control) were mixed in clean sterile test tubes. The volume of the sample mixture was adjusted to 500 µl by adding 30 µl of 10mM p-nitrophenyl-α-D-glucopyranoside (Sigma N1337) and 295µl buffer before incubation. The reaction sample mixtures were then kept for 12 minutes at 37 ^{0}C after which 3 ml of 50 mM NaOH were added in the reaction mixture. Absorbance reading of each sample reaction mixtures was noted at 410 nm. The percent α-glucosidase inhibition was calculated using the formula:

$$\alpha - \text{Glucosidase Inhibition (\%)} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

α-amylase inhibition assay

The inhibitory activity of *P. australis* to α-amylase enzyme was assessed *in vitro* using the procedures of Phoboo (2015) with slight modifications. Initially, solutions of alpha-amylase from porcine pancreas (0.5 mg/ml, Sigma A3176), 0.02 M Sodium-phosphate buffer, pH 6.9 with 0.006M NaCl and 1% starch solution were prepared. Varying concentrations of *P. australis* extract (with different phenolic concentrations) were prepared by dilution with water. To 50 µl of alpha-amylase solution, 25 µl of *P. australis* extracts or 25 µl buffer (for the control) were thoroughly mixed in clean sterile test tubes. The volume of the reaction mixtures was adjusted up to 250 µl by adding 175 µl phosphate buffer. This mixture was then added (at timed intervals) with 250 µl starch solution and was incubated for 20 min. After incubation, the reaction mixture was halted by adding 400 µl of DNS color reagent also at timed intervals. On the other hand, the blank used in the assay is consisted of 400 ml DNS reagent and 500 ml buffer. The reaction mixtures in test tubes were subjected to boiling water bath for about 5 min, cooled and were further diluted with 5 ml sterile distilled water. The absorbance reading of the control and sample mixtures were taken at a wavelength of 540 nm. The percent (%) inhibition was calculated using the equation:

$$\alpha - \text{Amylase Inhibition (\%)} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

Antibacterial activity

The bacterial test pathogens used in the study were obtained from the Philippine National Collection of Microorganisms (PNCM) of BIOTECH-UPLB. Four Gram-negative bacteria (*Pseudomonas aeruginosa* BIOTECH 1824, *Serratia marcescens* BIOTECH 1748, *Aeromonas hydrophila* BIOTECH 10089, and *Klebsiella pneumoniae* BIOTECH 1754) and four Gram-positive bacteria (*Listeria monocytogenes* BIOTECH 1958, *Bacillus cereus* BIOTECH 1509, *Staphylococcus aureus* BIOTECH 1823, and Methicillin-Resistant *Staphylococcus aureus* BIOTECH 10378) were tested against *P. australis* crude extract using microtiter plate dilution assay. Initially, these bacterial pathogens were grown using Luria Bertani (LB) Broth and was incubated for 24 hours at 37^{o}C with shaking. The purity as well as the viability of each bacterial test pathogens were regularly monitored by conducting regular morphological characterization and biochemical tests (Arguelles *et al.*, 2021a).

Microtiter plate dillution (two-fold serial dilution technique) assay was used to know the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *P. australis* extract (Arguelles *et al.*, 2021a). Briefly, 100 µl of the bacterial stock cultures were mixed with 100 µl of *P. australis* extract at different dilutions (1000 µg/ml - 7.8125 µg/ml) in a 96-well microtiter plate. The microtiter plate was incubated at 35℃ for 12 hours, after which the minimum inhibitory concentration (MIC) for each bacterium were recorded. MIC of *P. australis* extract is the minimum concentration of the seaweed extract that showed bacterial growth inhibition after 12 hours incubation period. On the other hand, minimum bactericidal activity (MBC) of *P. australis* extract was determined by inoculating a loopful of the sample from each MIC wells (that exhibited no visible bacterial growth) into freshly prepared tryptic soy agar. The plates were kept at 35℃ for 24 hours and were examined for bacterial growth for each specific dilution subculturing. Absence of bacterial colony growth would mean that the algal extract was bactericidal at that specific dilution (Arguelles, 2018; Arguelles *et al.*, 2021a).

Statistical analyses

The data obtained from the different experimental assays are expressed as means ± standard deviations (mean ± SD) of three replicates. The statistical tests for the linear correlation coefficient necessary in correlation analysis were determined using MS Office Excel 2016.

Results

Total phenolic content and extraction yield

Padina australis collected from the coastal area of General Nakar, Quezon was extracted using acidified methanol (1:30) with stirring for 1 hour using an ultrasonic bath. The collected crude extract of *P. australis* is brownish in color and was estimated to have a total yield extract of 11.57 ± 0.07%. On the other hand, the TPC of the algal extract was determined using Folin-Ciocalteu reagent and was expressed in milligram gallic acid equivalent per gram extract (mg GAE/g). The TPC of the algal extract was observed to be 13.85 ± 0.04 mg GAE/g. These results are presented in Table 1.

Table 1. Extraction yield and total phenolic content of *Padina australis* acidified methanolic extract

Sample	Extract	Extract Yield (%)	Total Phenolic Content (mg GAE/g)
Padina australis	Acidified Methanol	11.57 ± 0.07	13.85 ± 0.04

Antioxidant activity

The antioxidant activities of *Padina australis* were assessed by evaluating the scavenging activity against $ABTS^+$ free radical and copper reduction capacity of the crude extract. As shown in Table 2, the free radical scavenging property of the algal extract was found to cause inhibition of $ABTS^+$ free radicals in a dose-dependent manner. The computed effective concentration (IC_{50}) of *P. australis* extract is 138 μg/ml which is more potent as compared to the positive control, ascorbic acid, with IC_{50} value of 161 μg/ml.

Table 2. $ABTS^+$ radical scavenging activity and IC_{50} value of phenolics from *Padina australis* and ascorbic acid

Sample	Extract concentration (μg GAE/ml)					IC_{50}*
	35.0	70.0	105.0	140.0	175.0	
	$ABTS^+$ Inhibition (%)					
Padina australis	17.89 ± 1.00	30.85 ± 0.20	40.99 ± 0.20	50.42 ± 0.40	56.62 ± 0.40	138 μg/ml
	Concentration (μg/ml)					
	37.5	75.0	112.5	150.0	187.5	
	$ABTS^+$ Inhibition (%)					
Ascorbic Acid**	12.24 ± 0.80	23.21 ± 0.00	36.08 ± 0.30	47.40 ± 0.40	55.98 ± 0.20	161 μg/ml

*IC_{50} is the effective concentration that inhibits the activity of $ABTS^+$ (2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) cation radical by 50%. Computed by interpolation.

**A reference antioxidant.

Padina australis extract also exhibited copper ion reduction ability in a concentration-dependent manner. Table 3 shows the highest CUPRAC value of *P. australis* extract at 25 μg/ml concentration, in contrast with the standard, ascorbic acid, which showed highest CUPRAC value at phenolic concentration of 50 µg/ml. Comparison of the IC_{50} value showed that the seaweed extract showed a more potent antioxidant activity as compared to ascorbic acid (standard antioxidant) with IC_{50} value of 24.47 µg/ml and 46.46 µg/ml, respectively. The antioxidant activity observed in this study is similar to that obtained for $ABTS^+$ scavenging assay in which 175 µg/ml concentration showed the highest $ABTS^+$ free radical inhibition of 56.62%.

Table 3. Copper reduction antioxidant capacity (CUPRAC) and IC_{50} value of phenolics from *Padina australis* and ascorbic acid

Sample	**Extract concentration (µg GAE/ml)**					**IC_{50}***
	5.0	10.0	15.0	20.0	25.0	
	CUPRAC (Absorbance at 450 nm)					
Padina australis	0.118 ± 0.004	0.256 ± 0.006	0.372 ± 0.011	0.480 ± 0.006	0.579 ± 0.001	24.47 µg/ml
	Concentration (µg/ml)					
	10.0	20.0	30.0	40.0	50.0	
	CUPRAC (Absorbance at 450 nm)					
Ascorbic Acid**	0.114 ± 0.005	0.227 ± 0.000	0.334 ± 0.013	0.439± 0.013	0.534 ± 0.012	46.46 µg/ml

*IC_{50} is the effective concentration that gives CUPRAC value of 0.5 absorbance reading at 450 nm. Computed by interpolation.
**A reference antioxidant.

Correlation analysis between antioxidant activities and phenolic concentration of *P. australis* crude extract using CUPRAC assay and $ABTS^+$ free radical scavenging assay is presented in Table 4. The analysis exhibited a positive correlation between antioxidant ($ABTS^+$ radical scavenging and CUPRAC assays) capacity and the phenolic concentrations of the seaweed extract with R=0.9924 and R=0.9979, respectively.

Table 4. Correlation between phenolic content and antioxidant activities of *Padina australis* extract

Antioxidant Assay	**Regression Equation**	**Correlation Coefficient (R)**
$ABTS^+$ Radical Scavenging Assay	y = 0.2767x + 10.317	0.9924
Copper Reduction Antioxidant Capacity (CUPRAC) Assay	y = 0.0229x + 0.0172	0.9979

Tyrosinase inhibition activity

The capacity of *Padina australis* extract to inhibit tyrosinase enzyme was analyzed *in vitro* using mushroom tyrosinase (Table 5). The highest tyrosinase inhibition activity for *P. australis* extract was observed to be 86.94% at 75 µg/ml concentration. In addition, *Padina australis* extract exhibited potent tyrosinase inhibition activity with IC_{50} value of 32 µg/ml as compared to that obtained for the control (kojic acid) with IC_{50} of 101 µg/ml. Such results proved that *P. australis* extract is considered more effective than kojic acid and that the seaweed extract may contain bioactive compounds that has anti-melanogenic activities.

Table 5. Tyrosinase inhibition activity and IC_{50} value of phenolics from *Padina australis* and kojic acid

Sample	**Extract concentration (µg GAE/ml)**					**IC_{50}***
	15.0	30.0	45.0	60.0	75.0	
	Tyrosinase inhibition (%)					
Padina australis	31.33 ± 1.81	47.51 ± 0.46	65.70 ± 0.38	80.13 ± 3.91	86.94 ± 0.95	32.0 µg/ml
	Concentration (µg/ml)					
	50.0	100.0	150.0	200.0	250.0	
	Tyrosinase inhibition (%)					
Kojic Acid**	32.30 ± 1.02	49.75 ± 0.24	65.64 ± 2.38	72.86 ± 0.37	76.41 ± 0.43	101.0 µg/ml

*IC_{50} is the effective inhibitory concentration that inhibits tyrosinase activity by 50%. Computed by interpolation.
**A reference tyrosinase inhibitor and known whitening agent.

Elastase inhibition activity

The anti-wrinkling ability of *Padina australis* extract was evaluated *in vitro* via inhibition of elastase. Prepared concentrations of *P. australis* extract exhibited a dose-dependent elastase inhibition activity (Table 6). This would mean that the elastase inhibition activity of *P. australis* extract increases with an increase in the concentration of the algal extract (35.0 - 175.0 µg GAE/ml). The IC_{50} value of *P. australis* extract is 93 µg/ml which is considered more potent than that obtained from tocopherol (wherein 50% inhibition of elastase was not achieved at 2500 µg/ml concentration). This result suggests the potential of this organism as alternative source of active compounds with anti-wrinkling activities.

Table 6. Elastase inhibition activity and IC_{50} value of phenolics from *Padina australis* and tocopherol

Sample	**Extract concentration (µg GAE/ml)**					**IC_{50}***
	35.0	70.0	105.0	140.0	175.0	
	Elastase inhibition (%)					
Padina australis	15.48 ± 1.70	39.46 ± 1.95	56.04 ± 1.55	68.13 ± 1.25	77.68 ± 0.60	93.0 µg/ml
	Concentration (µg/ml)					
	500	1000	1500	2000	2500	
	Elastase inhibition (%)					
Tocopherol**	16.58 ± 0.19	19.35 ± 0.06	26.08 ± 1.13	31.03 ± 0.95	38.22 ± 0.37	>2500 µg/ml***

*IC_{50} is the effective concentration that inhibits elastase activity by 50%. Computed by interpolation
**A reference elastase inhibitor and known anti-wrinkling agent.
***IC_{50} was not determined because 50% inhibition was not achieved at 2500 µg/ml concentration.

Antidiabetic activities

The potential of *Padina australis* as alternative source of bioactive compounds with antidiabetic properties were assessed by measuring the ability of the algal extract to cause inhibition of α-amylase and α-glucosidase (known carbohydrate hydrolyzing enzymes). Result of the α-glucosidase inhibitory activity assay for *P. australis* extract is presented in Table 7. At phenolic extract concentration of 8.0 µg GAE/ml, the acidified methanolic extract of *P. australis* exhibited maximum inhibition activity of 74.79 ± 2.49% with an IC_{50} value of 5.9 µg/ml. On the other hand, acarbose, which served as the positive control, showed a computed IC_{50} value of 6771 µg/ml. In this assay, IC_{50} value for α-glucosidase inhibition of the algal crude extract is considered more potent than that of acarbose (standard antidiabetic drug).

Table 7. α-glucosidase inhibition and IC_{50} of phenolics from *Padina australis* in comparison to acarbose

Sample	**Extract concentration (µg GAE/ml)**					**IC_{50}***
	4.0	5.0	6.0	7.0	8.0	
	Alpha-glucosidase inhibition (%)					
Padina australis	9.28 ± 0.15	24.83 ± 0.78	53.52 ± 0.00	62.59 ± 0.05	74.79 ± 2.49	5.9 ug/ml
	Concentration (µg/ml)					
	2,000.0	4,000.0	6,000.0	8,000.0	10,000.0	
	Alpha-glucosidase inhibition (%)					
Acarbose**	17.96 ± 1.36	31.69* ± 1.22	45.32 ± 1.90	57.26 ± 0.49	62.35 ± 0.49	6771 ug/ml

* IC_{50} is the effective concentration that inhibits α-glucosidase activity by 50%.
**A reference α-glucosidase inhibitor and anti-diabetic drug.

The α-amylase inhibition activity of *P. australis* extract is shown in Table 8. Different concentration of the prepared seaweed crude extract exhibited a dose-dependent inhibition of α-amylase enzyme activity. *P. australis* extract showed high inhibitory activity against α-amylase at 75 μg GAE/ml with 85.41 ± 0.57% inhibition and a corresponding IC_{50} value (effective concentration) of 41 μg/ml. This IC_{50} value is conisidered more potent than that of acarbose, which gave an IC_{50} value of 103 μg/ml.

Table 8. α-amylase inhibition and IC_{50} of phenolics from *Padina australis* in comparison to acarbose

Sample	**Extract concentration (μg GAE/ml)**					**IC_{50}***
	15.0	30.0	45.0	60.0	75.0	
	Alpha-amylase inhibition (%)					
Padina australis	14.34 ± 0.75	29.77 ± 0.77	58.94 ± 0.09	69.34 ± 0.13	85.41 ± 0.57	41 ug/ml
	Concentration (μg/ml)					
	60.0	120.0	180.0	240.0	300.0	
	Alpha-amylase inhibition (%)					
Acarbose**	35.41 ± 0.30	55.86 ± 0.78	67.11 ± 0.65	74.80 ± 0.43	80.90 ± 0.74	103 ug/ml

* IC_{50} is the effective concentration that inhibits α-amylase activity by 50%.
**A reference α-amylase inhibitor and anti-diabetic drug.

Antibacterial activities

The potential of *P. australis* for antibacterial activity was done *in vitro* using microtiter plate dilution assay against eight medically important bacterial pathogens. The antibacterial activities of *P. australis* extract is shown in Table 9. *Padina australis* exhibited potent antibacterial activity against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* with MIC and MBC value of 125 and 250 μg/ml, respectively. On the other hand, Methicillin-Resistant *Staphylococcus aureus* and *Staphylococcus aureus* were also moderately inhibited by the seaweed exract with MIC and MBC value of 250 and 500 μg/ml, respectively. Moreover, no antibacterial activity was observed against *L. monocytogenes*, *B. cereus, S. marcescens*, and A. *hydrpophila*. The result of this study is the first documented report in the Philippines regarding antibacterial activities of *P. australis* against *Klebsiella pneumoniae, Pseudomonas aeruginosa Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus*.

Table 9. Antibacterial activities of *Padina australis* extract

Bacterial Pathogen	Minimum inhibitory concentration (µg/ml)	Minimum bactericidal concentration (µg/ml)
Gram-positive bacteria		
Methicillin-Resistant *Staphylococcus aureus* BIOTECH 10378	250.00	500.00
Staphylococcus aureus BIOTECH 1823	250.00	500.00
Listeria monocytogenes BIOTECH 1958	>1000.00	ND
Bacillus cereus BIOTECH 1509	>1000.00	ND
Gram-negative bacteria		
Pseudomonas aeruginosa BIOTECH 1824	125.00	250.00
Klebsiella pneumoniae BIOTECH 1754	125.00	250.00
Serratia marcescens BIOTECH 1748	>1000.00	ND
Aeromonas hydrophila BIOTECH 10089	>1000.00	ND

*ND = None Detected

Discussion

Extraction yield is the portion of the algal crude extract that can be utilized from the seaweed sample (Sanger *et al.*, 2019). *Padina australis* was extracted using acidified methanol as solvent for extraction. The crude extract of *P. australis* obtained from this study is brownish in color, which can be attributed to algal pigments such as xanthophylls, violaxanthin, carotenoids, fucoxanthin, and chlorophyll (Sanger *et al.*, 2019). The extraction yield of *P. australis* crude extract is 11.57 ± 0.07% which is higher as compared that obtained from methanol extracts of *Gracilaria salicornia* (3.56 ± 0.12%), *Halymenia durvilae* (6.34 ±0.43%) *Turbinaria decurrens* (5.72 ±0.23%) *Sargassum olygocystum* (4.95 ±0.03%), *Ulva rigida* (10.75 ±0.95%) *Bifucaria bifucarta* (10.85 ±0.70%), and *Enteromorpha intestinalis* (8.52 ±0.68%) from the coastal area of Indonesia and Morocco (Chernane *et al.*, 2014; Sanger *et al.*, 2019). Differences in the extraction yield of *P. australis* reported in this study as compared to previous studies from other seaweeds can be ascribed to several factors like solvent, sample particle size, method of extraction, and temperature used in the extraction protocol (Sivagnanam *et al.*, 2015). Hence, optimization of the extraction condition is recommended for large-scale production of active compounds from *P. australis*.

Seaweeds are rich natural sources of phenolic compounds which are known to have promising biological activities with important therapeutic application. The total phenolic content of *P. australis* is 13.85 ± 0.04 mg GAE/g. This result was comparable to other species of *Padina* such as *P. antillarum* and *P. pavonica* with TPC of 12.4 mg GAE/g and 7.06 mg PGE/g, respectively (Chew *et al.,* 2008; Abdelhamid *et al.,* 2018). On the other hand, Arguelles and Sapin, (2020a,c) showed that brown seaweeds such as *Turbinaria decurrens* and *Turbinaria ornata* have higher TPC, which are 27.84 ± 0.12 mg GAE/g and 18.50 ± 0.17 mg GAE/g, respectively. However, lower total phenolic content was observed for *Sargassum polycystum* (0.37 mg GAE/g) *Turbinaria conoides* (0.09 mg GAE/g) and *Sargassum ilicifolium* (4.86±0.07 mg GAE/g) (Boonchum *et al.,* 2011; Fu *et al.,* 2015; Arguelles, 2021b). Generally, the variation in the phenolic content of seaweed samples is influenced by several factors such as extraction protocol, particle size, as well as time and storage condition of the sample. In addition, the presence of substances that can interfere the extraction of the phenolic substances such as pigments, fats, and waxes can also affect the amount of the extracted phenolic compounds. Thus, it is important to take note that in the preparation of phenolic extracts form seaweed sample there is no single extraction protocol, since several studies are working with different extraction parameters (Mekinić *et al.,* 2019; Arguelles and Sapin, 2020b).

The antioxidant activities of *P. australis* crude extract were analyzed using $ABTS^+$ radical scavenging and copper reduction antioxidant capacity (CUPRAC) assays. Results showed that *P. australis* has potent antioxidant activities, more effective than the control (ascorbic acid). *Padina australis* was able to exhibit $ABTS^+$ radical scavenging activity which is more potent than those obtained from *Ulva intestinalis* and *Halimeda tuna* with IC_{50} of 1.50 mg/ml and 16.1 mg/ml, respectively (Srikong *et al.,* 2017; Sivaramakrishnan *et. al.,* 2017). However, Chakraborty *et al.,* (2013) exhibited that ethyl acetate extract of a red seaweed (*Halimeda musciformis*) possesses a more potent antioxidant activity, with IC_{50} value of 0.51 μg/ml. *Padina australis* extract also showed an effective copper reduction antioxidant capacity. The seaweed extract is comparatively more effective to that of *Turbinaria ornata* (IC_{50} value of 24.34 μg/ml) but is less potent than that of *Sargassum ilicifolium* with IC_{50} value of 11.19 μg/ml (Arguelles, 2021b; Arguelles and Sapin, 2020c). The results obtained in these antioxidant assays showed that *P. australis* have potent free radical scavenging activity and is capable of inhibiting oxidation via metal chelation mechanism. The antioxidant activities can be attributed to phenolic compounds such as phloroglucinol, bromophenols, and phlorotannins that are present in the seaweed extract. These phenolic compounds are considered potent antioxidants with excellent free radical scavenging activity capable of terminating oxidation process (Srikong *et al.,* 2017; Arguelles, 2021a). The present study also showed that the high total phenolic content of *P. australis* resulted in a potent antioxidant activity. Likewise previous studies demonstrated that seaweeds (such as *Codium intricatum*, *Ulva intestinalis* and *Turbinaria conoides*) showed maximum antioxidant activity at higher phenolic content (Srikong *et al.,* 2017; Ponnan *et al.,* 2017; Arguelles 2020). The correlation coefficient (R) between antioxidant activities (using $ABTS^+$ radical scavenging and CUPRAC assay) of *P. australis* and phenolic concentration are shown in Table 4. Results showed that a positive correlation exists suggesting that phenolic compounds contained in the algal extract play a crucial role in the potent free radical scavenging and metal ion chelating abilities exhibited by *P. australis* extract. This observation is similar to previous studies showing positive correlations between antioxidative properties and phenolic contents from other seaweed species like *Sirophysalis trinodis*, *Palisada perforata*, *Sargassum vulgare*, and *Sargassum angustifolium* (Pirian *et al.,* 2017; Arguelles *et al.,* 2019).

Tyrosinase and elastase are two important enzymes associated with skin aging which can cause severe hyperpigmentation and wrinkling. Tyrosinase is an enzyme important for melanin biosynthesis causing brown pigmentation of skin while elastase is a proteinase that degrades elastin (extracellular matrix proteins which provides elasticity to the connective tissues of skin). Thus, inhibition of elastase and tyrosinase activities is a useful method in the development of skin care products that address skin aging (Puspita *et al.,* 2017). In this investigation, the possibility of using *P. australis* extract as alternative source of active compounds for cosmetic application was evaluated via tyrosinase and elastase inhibition assays. The algal extract exhibited potent tyrosinase inhibition property with IC_{50} value of 32.0 μg/ml and is considered more effective as compared to the control (kojic acid) with IC_{50} value of 101 μg/ml. The antityrosinase activity of *P. australis* extract is also more effective than other seaweeds reported such as *Turbinaria conoides* (IC_{50} = 188.85 μg/ml), *Sargassum siliquosum* (IC_{50} = 65.0 μg/ml), and *Asparagopsis armata* (IC_{50} = 153.98 ppm) (Sari *et al.,* 2019; Lee *et al.,* 2020; Arguelles and Sapin, 2020b). The capacity of *P. australis* extract to inhibit elastase was done *in vitro* (Table 6). *Padina australis* extract exhibited a dose-dependent elastase inhibition activity. The IC_{50} value of *P. australis* extract is 93 μg/ml which is more effective than that obtained from tocopherol (standard elastase inhibitor). In addition, elastase inhibition activity of *P. australis* extract is considered more potent than that obtained for *Lobophora variegata* (IC_{50} value = >250.0 µg/ml) but is less effective to other brown seaweeds such as *Fucus spiralis* (IC_{50} value = 3.0 µg/ml) and *Sargassum muticum* (IC_{50} value = 21.6-32.8 µg/ml) (Puspita *et al.*, 2017; Freitas *et al.*, 2020; Susano *et al.*, 2021). Differences in the elastase and tyrosinase inhibition properties of seaweed extracts is caused mainly by environmental (seasonal differences and habitat characteristics) and physiological factors such as age, harvesting condition and strain differences in algal species (Arguelles and Sapin, 2020b). Seaweed-derived phenolic compounds such as phlorotannins, dieckol, and 7-Phloroekol are documented to have naturally derived anti-aging and anti-wrinkling active ingredient useful for cosmeceutical application. These phenolic compounds can imitate the substrates of the target enzmes (tyrosinase and elastase) causing competitive inhibition and inactivation of the enzymes (Baek *et al.*, 2021).

The α-amylase and α-glucosidase are two known carbohydrate metabolism enzymes in the digestive system that controls the occurrence of hyperglycemia. α-amylase hydrolyzes starch, glycogen and oligosaccharides via degradation of the α-1,4-glucosidic bonds. On the other hand, α-glucosidase degrades disaccharides into smaller sugar units for efficient intestinal absorption. Thus, inhibition of these key enzymes can control hyperglycemia by limiting glucose absorption (Hwang *et al.*, 2015). *Padina australis* showed potent inhibition of these enzymes exhibiting IC_{50} values of 41.0 µg/ml and 5.9 µg/ml for α-amylase and α-glucosidase, respectively. These IC_{50} values are considered more effective than those observed for the control (acarbose) for α-amylase and α-glucosidase (Tables 7 and 8). The crude extract of *P. australis* efficiently inhibits the activity of the carbohydrate degrading enzymes and showed a concentration dependent increase in the percentage of enzyme inhibition. Thus, suggesting that *P. australis* can be use in the control and regulation of postprandial hyperglycemia and possibly for treatment of diabetes. These results are further supported by earlier studies from seaweed extracts of *Sargassum hystrix* (α-amylase IC_{50} = 0:58 ± 0:01 mg/ml; α-glucosidase IC_{50} = 0:59 ± 0:02 mg/ml), *Ascophyllum nodosum* (α-amylase IC_{50}: 0.1 µg/ml; α-glucosidase enzymes IC_{50} = 19 µg/ml), and *Gracillaria edulis* (α-amylase IC_{50} = 279.48 µg/ml and α-glucosidase IC_{50} = 87.92 µg/ml) which have been documented to show potent hypoglycemic effects via inhibition of α-amylase and α-glucosidase enzymes (Nwosu *et al.*, 2011; Husni *et al.*, 2018; Gunathilaka *et al.*, 2019). In addition, correlation analysis between phenolic concentration of *P. australis* crude extract and antidiabetic activities via α-amylase and α-glucosidase inhibition assays showed a positive correlation suggesting the potential role of phenolic compounds in the antidiabetic properties of the seaweed extract. Likewise, such correlation was also observed by Gunathilaka *et al.,* (2019) and Hwang *et al.,* (2015) where they associated the potent α-amylase and α-glucosidase inhibition activities of various seaweed species crude extracts to their high phenolic content. Brown seaweeds have phenolic compounds (such as phlorotannins, bromophenols, and dihydrobenzoic acid) that can form protein complexes (to α-amylase and α-glucosidase), causing these enzymes to precipitate and have structural alteration in combination with loss of biological activities (Firdaus *et al.,* 2015). In addition, hydroxyl groups found in phenolic compounds can also react with these enzymes which promotes inhibitory activities of these bioactive compounds (Kim *et al.,* 2008). This confirms the observed potent α-amylase and α-glucosidase inhibitions exhibited by *P. australis* crude phenolic extract in the current study. The results of these assays suggest the promising use of this seaweed as an alternative source of natural antidiabetic drugs for treatment of diabetes.

Seaweeds are well known producers of new bioactive compounds that have potential pharmaceutical value, including antibacterial activity (Arguelles and Sapin, 2020a). In this investigation, the antibacterial activity of *P. australis* was evaluated against eight bacterial pathogens using microtiter plate dilution assay. Potent antibacterial activities were observed against *K. pneumoniae* and *P. aeruginosa* both with MIC and MBC of 125 μg/ml and 250 μg/ml, respectively. The antibacterial activity of *P. australis* against *P. aeruginosa* is more potent than those observed by Chong *et al.,* (2011) and Sasidharan *et al.,* (2010) from methanolic extracts of *P. australis* (MIC = 0.26 mg/ml) and *Gracillaria changii* (MIC=6.25 mg/ml). In addition, *P. australis* was able to inhibit Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus aureus* both with MIC and MBC of 250 μg/ml and 500 μg/ml, respectively. This antibacterial activity against *S. aureus* is considered more effective from those observed by Mashjoor *et al.,* (2016) from methanol extracts of *Ulva flexuosa* (MIC = 3.75 mg/ml), *Padina antillarum* (MIC = 7.5 mg/ml), and *Padina boergeseni* (MIC = 15 mg/ml). However, it is less effective from ethyl acetate extract of *Enteromorpha prolifera* (MIC = 1 μg/ml) and petroleum ether extract of *Padina pavonica* (MIC = 1.25 μg/ml) taken from the coastal regions of Morocco (Rhimou *et al.,* 2010). On the other hand, no antibacterial activities were observed from *P. australis* extract against *L. monocytogenes, B. cereus*, *S. marcescens* and *A. hydrophila*. This observation is contrary to that observed by Chong *et al.,* (2011) from methanol extract of *P. australis* that showed antibacterial activity against *B. cereus* with MIC value of 0.21 mg/ml. Such variations in the antibacterial activities of the seaweed extract may be due to differences in the solvents (extractant) and extraction protocols, as well as the active compounds present in the algal extract which was collected from different geographical locations and seasons (Arguelles and Sapin, 2020b). Phenolic compounds (such as phenolic acids, tannins, flavonoids, phloroglucinol, and lignans) from seaweeds are associated with a strong antibacterial activities. These compounds are almost present exclusively in brown seaweeds and are known to cause bacterial cell lysis of several medically-important bacterial pathogens (Arguelles, 2021b).

In the Philippines, limited scientific studies are available showing the potential biological properties of *P. australis*. Thus, this study is the first documented report in the country about the antioxidant, antibacterial, antidiabetic, tyrosinase and elastase inhibition activities of this seaweed. *Padina australis* contains a high concentration of polyphenols and exhibited potent antioxidant, α-amylase, α-glucosidase, tyrosinase, elastase inhibition as well as antibacterial activities with direct relevance to pharmaceutical application. It is recommended that investigation on the purification, isolation, identification, and elucidation of the chemical structures of the active compounds of *P. australis* crude extract should be done to further understand the reaction mechanisms involve in other biological activities *in vivo*. *Padina* is a diverse group of marine algal resource that is abundantly available in the coastal areas of the Philippines, hence the availability and sustainability of this seaweed for commercial and industrial use.

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