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ORIGINAL RESEARCH

Phytochemical composition, antioxidant, and antibacterial activity of the Philippine marine green alga (*Ulva pertusa*)

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Abstract The phytochemical constituents, total phenolic and flavonoid contents, antioxidant and antimicrobial activities of the ethanolic extract of the marine green alga *Ulva pertusa* collected from floating fish net cages off the coast of Guimaras island, Philippines, were investigated. Qualitative phytochemical analysis revealed the presence of bioactive compounds including alkaloids, flavonoids, phenols, saponins, tannins, and terpenoids. Quantitatively, total phenolic and flavonoid contents of the extract were 20.54±2.08 mg gallic acid equivalent (GAE) g^{-1} and 539.07±6.36 mg rutin equivalent (RE) g^{-1}, respectively. The antioxidant activity of the extract using 1,1-diphenyl-1-picrylhydrazyl (DPPH) assay exhibited a concentration-dependent radical scavenging capacity. Additionally, the ethanolic extract inhibited the growth of *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC25922, *Streptococcus agalactiae*, *Aeromonas hydrophila*, *A. sobria,* and *Vibrio harveyi* in a concentration-dependent manner with strong inhibitory activity at 100 mg mL^{-1} concentration. Taken together, *U. pertusa* contains bioactive compounds that possess potent antibacterial activity and remarkable antioxidant capacities. These substances are promising candidates that may be utilized in the synthesis of novel drugs.

Keywords *Ulva pertusa* . Antimicrobial activity . Antioxidant . *Streptococcus agalactiae* . *Aeromonas* species

Introduction

Being one of the primary producers in the ocean, the role that seaweeds play has been regarded crucial in the marine ecosystem. Seaweeds are potential renewable living resources that are not only utilized as human food but as well as animal feed and plant fertilizer in many parts of the world (Ferdouse et al. 2018). About 6000 species of seaweeds have by far been identified and accordingly grouped into 3 different classes, i.e. green (Chlorophytes), brown (Phaeophytes), and red (Rhodophytes) algae (Chandini et al.

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2008). The global seaweed production had doubled from 14.6 million tonnes in 2005 to 30.4 million tonnes in 2015, with 29.4 million tonnes and 1.1 million tonnes generated from the aquaculture and capture sectors, respectively (Ferdouse et al. 2018). There has been a growing interest with regard to mass propagation of seaweeds because majority of the maricultured seaweeds have been found to possess medicinal activities (Pérez et al. 2016). Several bioactive substances isolated from green, brown, and red marine seaweeds including among others, polysaccharides, proteins, fatty acids, phenolics, and terpenes are important as they have been documented to have significant antibacterial, antiviral, anticancer, anti-inflammatory, and anti-oxidative properties (Devi et al. 2008; Satpati and Pal 2011; Pérez et al. 2016; Thanigaivel et al. 2016; Li et al. 2018b).

Microorganisms have developed novel strategies to evade the action of antibiotics, leading to the emergence of multiple drug-resistant bacterial strains. As fish inevitably becomes susceptible to various bacterial infectious diseases particularly when reared in high stocking densities, occurrence of unwarranted bacterial disease outbreaks has been recognized as one of the major contributors responsible for elevated mortality rates, reduced productivity efficiency, and resultant high economic losses to the fish farmers. The application of antibiotics to treat bacterial infections has by far been intensively practiced by fish farmers as mitigating measure aimed at controlling if not eradicating the disease. However, over the past several years, the indiscriminate use of antibiotics has unfortunately led to the emergence of drug-resistant bacteria. For example, emergence of multiple drug-resistant bacterial strains implicated in diseases of various pond-cultured fish species like tilapias and milkfish has been reported in *Streptococcus iniae, S. agalactiae, Aeromonas hydrophila, A. sobria, A. salmonicida, Edwardsiella tarda, E. ictaluri, Vibrio harveyi,* and *V. anguillarum* among others (Martinez et al. 2009; Cabello et al. 2013; Lo et al. 2014). It should be noted that these fish bacterial pathogens have been implicated in human diseases (food-borne or zoonotic) thereby making the aquaculture products as potential risk to the consumers (Cabello et al. 2013). Decreased efficacy and resistance of bacterial fish pathogens to commonly used antibiotics necessitates the development of new alternatives.

In recent years, seaweed extracts have been widely utilized for the prevention of bacterial and viral diseases in aquatic animals (Bansemir et al. 2006; Li et al. 2018a). In particular, seaweed extracts have been found to exhibit marked antibacterial activity against fish pathogenic bacteria (Bansemir et al. 2006; Cavallo et al. 2013; Thanigaivel et al. 2016). The green alga, *Ulva pertussa*, a light green seaweed typically composed of foliaceus blade with papery thin undulating margin, is a fast-growing seaweed that is typically opportunistic for space and nutrient uptake (Hurtado et al. 2006). *Ulva* is commonly found in the intertidal zones and labeled as nuisance species as they could over proliferate producing blooms or 'green tide' (Largo et al. 2004). Nevertheless, *Ulva* is not grown commercially outside Asia because there is no market for it (Bolton et al. 2009). However, it should be noted that *U. pertusa* is abundant in vitamins, trace elements, dietary fibers, and nutritiously low in calorie (Benjama and Payap 2011). Additionally, various bioactive compounds extracted from *U. pertusa* have been reported to have potent antibacterial (Choi et al. 2014), antiviral (Sun et al. 2018), and antioxidant (Choi et al. 2011) properties. For example, the sulfated polysaccharide extracted from *U. pertusa* has been shown to have powerful antioxidant activities, including scavenging ability of the superoxide and hydroxyl radicals, chelating ability of iron ion, and reducing power (Qi et al. 2005). While various studies delving on antioxidant, antibacterial, and antiviral properties of the different classes of seaweeds and concomitantly their wide application in food, pharmaceutical, and aquaculture industry have been comprehensively conducted in some seaweed-producing countries around the world, many types of wild and cultured seaweeds, particularly the green seaweed *U. pertusa*, in the Philippines have by far been unexplored. It is in this light that the current study was conducted to evaluate the phytochemical composition, antioxidant, and antibacterial activity of the ethanolic extract of the green alga (*U. pertusa*) collected from the floating net cages used to rear pompano (*Trachinotus blochii*) in Igang Marine Station (IMS), Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC/AQD) located in Guimaras island, Philippines. Resolute results generated from this study would plausibly strengthen the potentiality of *U. pertusa* extract as a novel and cost-effective antioxidant and antibacterial agent against pathogenic bacteria, particularly those involved in zoonosis, and undoubtedly, would be a substantial springboard that could spur further investigations on the isolation, purification, and identification of some of its yet unidentified bioactive compounds.

Materials and methods

Chemicals used

2,2-Diphenyl-1-picrylhydrazyly (DPPH), ascorbic acid, gallic acid, sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3$), and rutin were purchased from Sigma Aldrich (Philippines). Folin-Ciocalteu's phenol reagent and hydrogen peroxide, methanol, and dimethylsulfoxide (DMSO) were purchased from Merck (Philippines). All other solvents and chemicals used were of analytical grade.

Seaweed material

The green seaweed (*U. pertusa*) growing on the nets of grow-out floating cages used to rear pompano (*T. blochii*) broodstocks in SEAFDEC/AQD's Igang Marine Station (IMS), were collected in April and May wherein the ambient daytime water temperature ranged from 29-32 ºC. The collected seaweed samples used in the different experiments were authenticated by SEAFDEC/AQD's seaweed expert. The seaweed samples collected were thoroughly washed with sterile seawater followed by sterile distilled water. The washed samples were then stored in sterile plastic bags and transported in an iced condition to SEAFDEC/AQD's Fish Health Laboratory in Tigbauan, Iloilo within 1 hour after sampling. The seaweed samples were shade dried (38±2 ºC) in a drier for about 24 to 36 h, cut into small pieces and powdered using a mixer grinder. The powdered samples were collected in sterile amber bottles and stored at –20ºC until used.

Preparation of *U. pertusa* extract

The powdered seaweed was extracted with ethanol following a modified method adapted from (Li et al. 2018a). Briefly, 150 g of the powdered dried seaweed was soaked in 450 ml of 80% ethanol and allowed to stand at room temperature (28ºC) for 72 hours. The mixture was shaken at regular intervals during the course of the experiment. The extract was then filtered with sterile Whatman filter paper No. 1 (320 mm, 11 µm). The extraction procedure was repeated once more for another 72 hours, after which the two filtrates were combined and concentrated under reduced pressure at 45ºC using a rotary evaporator. The dried extracts were stored at –20ºC until needed for various analysis.

Phytochemical screening

The ethanolic extract of *U. pertusa* was subjected to various tests to detect the presence of phytochemical compounds following standard procedures as described by Harborne (1998) and Evans (2009). The qualitative detection of alkaloids present in *U. pertusa* ethanolic extract was carried out using Mayer's and Wagner's test. Additionally, the presence flavonoids (Shinoda test), glycosides (Keller-Killiani test), phenols and tannins (ferric chloride test), saponin (foam test), and terpenoids (Salkowski test), were likewise qualitatively examined.

Total phenolic content

The total soluble phenolic compounds in the *U. pertusa* extract were estimated with Folin-Ciocalteu reagent using gallic acid as standard following the method of Singleton et al. (1999) as described by Stankovic et al. (2011). The ethanolic solution of the extract in the concentration of 1mg mL^{-1} was used in the analysis. Briefly, 0.5 mL of the ethanolic extract was mixed with 2.5 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 mL of 7.5% $NaHCO_3$. The reaction mixture was mixed thoroughly and allowed to stand for 45 min at 45°C after which, the absorbance was measured at 765 nm versus blank sample using a spectrophotometer (Shimadzu, Japan). Samples were prepared in triplicate for each analysis and the mean value of the absorbance was accordingly obtained. The total phenolic content was determined using a standard curve with gallic acid and the result was expressed as mg gallic acid equivalent per gram (mg GAE g^{-1}) of extract.

Total flavonoid content

The total flavonoid content of *U. pertusa* extract was determined spectrophotometrically following a standard method (Quettier-Deleu et al. 2000). Briefly, 1 mL of 2% $AlCl_3$ reagent was mixed with the same volume of the *U. pertusa* ethanolic extract (1 mg mL^{-1}). After allowing the mixture to be incubated for 1 hour at room temperature (28ºC), absorption readings at 415 nm were recorded against a blank (ethanol) sample. The samples were prepared in triplicate for each analysis and the mean value of the absorbance was generated. The total flavonoid content was determined using a standard curve with rutin and the result was expressed as mg rutin equivalent per gram (mg RE g^{-1}) of extract.

DPPH radical scavenging activity

The ability of *U. pertusa* extract to scavenge 1,1-diphenyl-1-picrylhydrazyl (DPPH) free radicals was performed following the method described by Cho et al. (2010) with slight modifications. Briefly, the stock solution of extracts was prepared in ethanol to obtain concentrations of 3.0, 2.5, 2.0, 1.0, 1.50, 0.75, 0.50, and 0.25 mg mL^{-1}. One hundred microliter of each diluted solution was mixed with 100 µL of 0.1 mM DPPH prepared with ethanol. The solution was then incubated in the dark at room temperature (28ºC) for 30 minutes, after which the absorbance was measured at 515 nm using a microplate reader. Ethanol was used as blank while 100 µL ethanol mixed with 100 µL DPPH served as control. Additionally, ascorbic acid (20 µg mL^{-1}) (Vitamin C) standard was used as positive reference (Cho et al. 2010). The percentage of scavenged DPPH was calculated using the following equation:

$$\text{DPPH Scavenging activity (\%)} = ([A_{Con} - A_{Test}]/A_{con}) \times 100$$

Where A_{Con} is the absorbance of the control and A_{Test} is the absorbance of the sample. The half-maximal inhibitory concentration (IC_{50}) was calculated by linear regression analysis and expressed as mean of three determinations.

Antibacterial assay

Bacterial strains

Streptococcus agalactiae isolated from the kidney (isolate code: TKG510208) and spleen (TMD10206) of tilapia with streptococcal infection (Pakingking unpublished study), *Aeromonas hydrophila* isolated from the gills (TI201301) (Pakingking et al. 2020) and kidney (P1261308) (Albances 2015) of tilapia (*Oreochromis niloticus*) with aeromonad septicemia, *A. sobria* (TI2013025) isolated from the gills of *O. niloticus* (Pakingking et al. 2020), and *Vibrio harveyi* (TbE090) isolated from diseased pompano (*T. blochii*) (Pakingking et al. 2018) were used in the antibacterial assay. Additionally, *Staphylococcus aureus* ATCC25923 (gram-positive) and *Escherichia coli* ATCC25922 (gram-negative) were used as reference strains. All microorganisms used in the assay were maintained in trypticase soy broth (TSB; Merck) supplemented with 15% glycerol at –80ºC.

Antibacterial screening

The antibacterial activity of the seaweed extract was examined using the modified agar well diffusion method of Perez et al. (1990) as described by Mattana et al. (2010). Briefly, the seaweed extract was dissolved in 3% DMSO to obtain an initial concentration of 1000 mg mL^{-1} and sterilized by filtration through 0.45 µm membrane filter (Millipore). All tests were conducted in triplicate using different concentrations of the seaweed extracts diluted in 3% DMSO. Amoxicillin (0.02 mg mL^{-1}) was used as the standard antimicrobial agent.

Agar well diffusion method

Agar cultures of the bacteria used in the antibacterial assay were prepared as described by Pakingking

Table 1 Qualitative phytochemical screening of the *Ulva pertusa* ethanolic extract

Phytochemical compound	Test	Result		
		Aliquot 1	Aliquot 2	Aliquot 3
Alkaloids	Mayer's and Wagner's test	+	+	+
Flavonoids	Shinoda test	+	+	+
Glycosides	Keller-Killiani test	−	−	−
Phenols	Ferric chloride test	+	+	+
Saponin	Foam test	+	+	+
Tannins	Ferric chloride test	+	+	+
Terpenoids	Salkowski test	+	+	+

(+) = present; (−) = absent

et al. (2015). Briefly, the bacterial isolates were inoculated in TSB and incubated for 18-24 h at 35ºC. The concentration of the cultures was standardized by matching the turbidity with 0.5 McFarland standard using sterile normal saline solution (NSS) to obtain approximately 1×10^8 colony forming units per mL (CFU mL^{-1}). The prepared bacterial suspensions were then swabbed on the surface of Mueller-Hinton agar (MHA; Merck) plates, i.e. 25 mL of solidified MHA per plate, and subsequently punched with 7 mm diameter wells at appropriate distances apart using sterile cork borer. Each well was filled with 100 µL of the seaweed extract with varying concentrations ranging from 3.125 to 100 mg mL^{-1} (Table 3). Concomitantly, wells filled with 100 µL of amoxicillin (0.02 mg mL^{-1}) and 3% DMSO served as positive and negative controls, respectively. Plates were incubated at 35ºC for 24 h. After incubation, the plates were retrieved and the zones of growth inhibition surrounding the wells were recorded. Clear zones around the wells indicated the presence of antibacterial activity. All experiments were conducted in triplicate and one-way ANOVA was employed to compare the mean values of each treatment. Significant differences among the bacterial isolates tested per treatment or concentration of *U. pertusa* extract were compared by Duncan test ($P < 0.05$).

Results

Yield of *U. pertusa* extract

The quantity of the crude *U. pertusa* ethanolic extract obtained in the current study was 20.5±0.7 g which is equivalent to a yield of 13.7±0.3 %. This result comparatively concurs with the previous report of Li et al. (2018a) wherein these authors obtained a yield of approximately 12.9% from a 30 g powdered *U.* pertusa using 85% ethanol as extractant.

Phytochemical screening

As shown in Table 1, qualitative phytochemical analysis of the different aliquots of the crude *U. pertusa* ethanolic extract revealed the presence of different bioactive compounds including alkaloids, flavonoids, phenols, saponins, tannins, and terpenoids. However, all the representative aliquots examined were negative for the detection of glycosides.

Total phenolic content

As shown in Table 2, the total phenolic compounds quantified in the three aliquots of *U. pertusa* ethanolic extract ranged from 19.33±0.29 to 22.95±0.40 mg GAE g^{-1} with a mean of 20.54±2.08 mg GAE g^{-1}.

Total flavonoid content

The total flavonoid content of the crude *U. pertusa* ethanolic extract was determined with reference to the standard rutin and expressed as its equivalent (mg RE g^{-1}). The total flavonoids present in the three aliquots of *U. pertusa* ethanolic extract examined ranged from 533.69±23.03 to 546.09±6.83 mg RE g^{-1} with a mean

Table 2 Total phenolic and flavonoid contents of the *U. pertusa* ethanolic extract

Test	Result			
	Aliquot 1	Aliquot 2	Aliquot 3	Mean
Total phenolics content (mg gallic acid equivalent [GAE] g^{-1} of extract)	22.95±0.40	19.35±1.72	19.33±0.29	20.54±2.08
Total flavonoids content (mg rutin equivalent [RE] g^{-1} of extract)	533.69±23.03	546.09±6.83	537.42±24.81	539.07±6.36

Data are presented as Mean ± SD

Table 3 Antimicrobial activity of the *U. pertusa* ethanolic extract against selected bacterial fish pathogens

Microorganism	Inhibition Zone (mm ±SD)							
	Amoxicillin 0.02 mg mL^{-1}	DMSO	100 mg mL^{-1}	50 mg mL^{-1}	25 mg mL^{-1}	12.5 mg mL^{-1}	6.25 mg mL^{-1}	3.125 mg mL^{-1}
Gram positive								
Staphylococcus aureus ATCC25923	29.67±0.58	–	30.67±0.58^{a}	26.67±0.58^{a}	24.00±00^{a}	19.33±0.58^{a}	10.67±0.58^{a}	–
Streptococcus agalactiae TKG510208	21.67±0.58	–	17.67±0.58^{c}	13.17±0.29^{b}	10.33±0.58^{b}	8.00±0.00^{b}	–	–
Streptococcus agalactiae TMD10206	21.33±0.58	–	18.17±0.29^{bc}	12.83±0.29^{b}	9.83±0.29^{b}	8.00±0.00^{b}	–	–
Gram negative								
Escherichia coli ATCC25922	11.33±0.58	–	14.67±0.58^{d}	9.50±0.50^{c}	–	–	–	–
Aeromonas hydrophila TI201301	–	–	19.33±0.58^{b}	10.00±0.00^{c}	–	–	–	–
Aeromonas hydrophila P1261308	–	–	20.00±0.00^{b}	10.00±0.00^{c}	–	–	–	–
Aeromonas sobria TI2013025	–	–	17.67±0.58^{c}	10.33±0.58^{c}	–	–	–	–
Vibrio harveyi TbE0901	–	–	9.00±0.00^{e}	–	–	–	–	–

(–) no zone of inhibition. Data are presented as Mean ± SD. Values with different superscripts $^{a, b, c, d, e}$ within each column are significantly different as determined by Duncan test ($P < 0.05$)

of 539.07±6.36 mg RE g^{-1} (Table 2).

DPPH radical scavenging activity

The DPPH radical scavenging activity of the *U. pertusa* ethanolic extract is shown in Fig. 1. The *U. pertusa* ethanolic extract exhibited a concentration-dependent DPPH radical scavenging capacities from 0.25 to 3 mg mL^{-1}, such that the scavenging activities recorded correspondingly increased from 17±0.2 to 80±0.4 %, respectively. Accordingly, the computed IC_{50} of the *U. pertusa* ethanolic extract was 1.53±0.012 mg mL^{-1}. The scavenging activity of the *U. pertusa* extract was comparable to the scavenging capacity of our positive control (ascorbic acid).

Antibacterial activity

To better elucidate the antibacterial activity of the *U. pertusa* ethanolic extract, the agar well diffusion method was employed. All of the 3% DMSO control wells did not produce any zones of inhibition on any of the bacterial strains examined, while the antibiotic control wells produced zones of inhibition of 29.67±0.58 for *S. aureus* ATCC25923. As shown in Fig. 2 and Table 3, the *U. pertusa* ethanolic extract possesses potent antibacterial activity against *S. aureus* ATCC25923 as supported by a marked inhibition zone of 30.67±0.58 mm produced after a 24h exposure to 100 mg mL^{-1} of the extract. Concomitantly, a decreasing trend parallel to the decreasing concentrations of the extract was evidently noted such that at 6.25 mg mL^{-1}, the zone of inhibition that was produced was only 10.67±0.58 mm. Notably, the extract exhibited antimicrobial activity against *E. coli*, *A. hydrophila* and *A. sobria*. The antibacterial activity induced by the extract at 100 mg mL^{-1} against *S. agalactiae* and *Aeromonas* spp. was not significantly different ($P < 0.05$) whereas, a markedly lower activity against *V. harveyi* was noted (Table 3).

Discussion

Concordant to the previous phytochemical studies on green seaweed extracts (Cho et al. 2010; Wang et al.

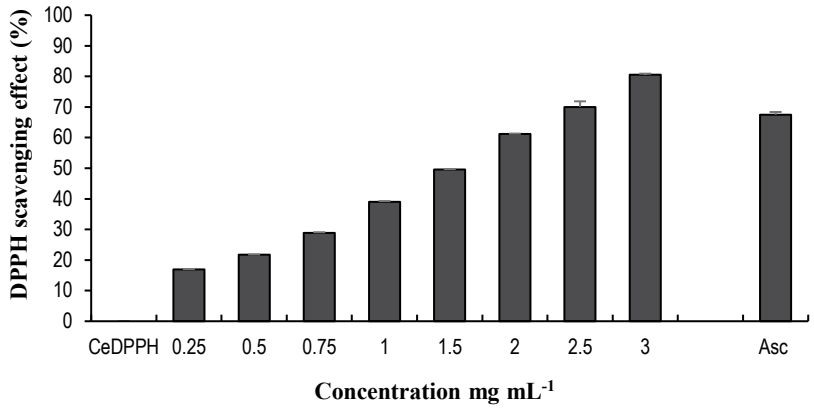

Fig. 1 DPPH (1,1-Diphenyl-1-picrylhydrazyl) radical scavenging activity of the *U. pertusa* ethanolic extract. Ethanol + DPPH (CeDPPH) and ascorbic acid (Asc) were used as negative and positive controls, respectively.

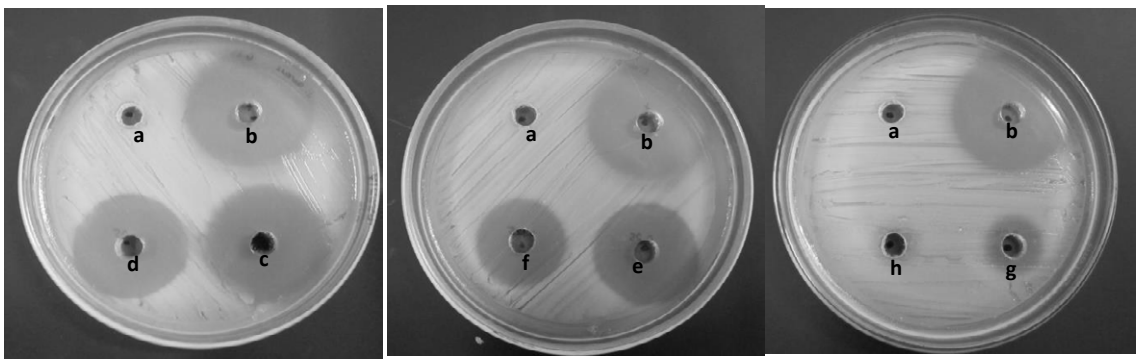

Fig. 2 Representative plates for the agar well diffusion assay showing zones of inhibition produced by the varying concentrations of the *U. pertusa* ethanolic extract (UpEE) on *S. aureus* ATCC25923. Negative control (DMSO) (a); Amoxicillin 0.02 mg mL^{-1} (b); 100 mg mL^{-1} UpEE (c); 50 mg mL^{-1} UpEE (d); 25 mg mL^{-1} UpEE (e); 12.5 mg mL^{-1} UpEE (f); 6.25 mg mL^{-1} UpEE (g); 3.125 mg mL^{-1} UpEE (h).

2010), our current data likewise revealed the presence of different bioactive compounds in the *U. pertusa* ethanolic extract including alkaloids, flavonoids, phenols, saponins, tannins, and terpenoids. Bioactive compounds stored in seaweeds possess antioxidant and antibacterial activities that can be utilized as alternative to treat bacterial infections in fish, particularly those bacterial pathogens implicated in zoonosis (Vatsos and Rebours 2015; Thanigaivel et al. 2016). These bioactive compounds detected in *U. pertusa* extract have been previously documented to confer resistance in opposition to microbial pathogens (Cho et al. 2010; Choi et al. 2011, 2014) and this could be accountable for the exposition of antioxidant and antibacterial activities of the extract in the present study. It should be noted that secondary metabolites stored in plants like terpenoids have been documented to have antiviral, antimalarial, antibacterial, and anti-inflammatory properties (Mahato and Sen 1997). Alkaloids, on the other hand, have been reported to have a wide range of pharmacological potentials like antiasthma, antimalarial, and anticancer activities (Thawabteh et al. 2019). Moreover, saponins have been identified to possess antidiabetic, antifungal, and anti-inflammatory properties (Feroz 2018). Additionally, tannins are polyphenolic compounds found to exhibit antioxidant, antimicrobial, and anti-inflammatory properties (Macáková et al. 2014).

Phenolic compounds are ubiquitous secondary metabolites commonly found in plants and have been documented to have several biological activities including antioxidant and antibacterial activities (Kuda et al. 2005). Accordingly, the Folin-Ciocalteu method was used to examine the total phenolic content of the *U. pertusa* extract. Folin-Ciocalteu reagent determines the total phenols as evidenced by the production of a blue color indicative of the reduction of the yellow heteropoly phosphomolydate-tungstate anions (Stankovic et al. 2011). The total phenolic content of the *U. pertusa* ethanolic extract in the current study was significantly higher compared to the values previously reported for ethanolic extracts of *U. pertusa*

and another green seaweed *Capsosiphon fulvescens* (Cho et al. 2010). However, current values were lower than those of the ethanolic extracts reported from another green seaweeds *Chaetomorpha monilifera* and *Enteromorpha compressa* (Cho et al. 2010). Although reports have indicated that phenolic content was found to be higher in ethanolic extracts due to its polarity, the differences in the total phenolic contents of the *U. pertusa* ethanolic extract between our current and previous studies may be attributed to the extraction protocol employed and viably, influence of various environmental factors on the phenolic contents (Kuda et al. 2005; Wang et al. 2010). Flavonoids, a group of secondary metabolites that refers to a class of naturally occurring polyphenols, are also commonly found in plants. They are typically accountable for taste, color, disablement of fat oxidation and prevention of enzymes and degradation of vitamins in food. Flavonoids have been reported to have anti-oxidative activity, free-radical scavenging capacity, coronary heart disease prevention, anticancer activity, and some have been found to exhibit potential for anti-human immunodeficiency virus functions (Yao et al. 2004). The mean total flavonoid content of the *U. pertusa* ethanolic extract obtained in the current study was significantly higher than the previous report of Farasat et al. (2014) for the methanolic extract of the green seaweeds *Ulva clathrata* and *U. intestinalis*. The differences noted between the data generated in the current study and the previous report of Farasat et al. (2014) may be plausibly attributed to the method of extraction used and possibly due to marked changes in the chemical constituents of seaweeds with the change of seasons and environmental conditions such as variation in physicochemical parameters of the rearing water (Kuda et al. 2005; Wang et al. 2010; Farasat et al. 2014). Because phenolic compounds including flavonoids which are commonly found in plants have been generally proven to be effective free radical scavengers and antioxidants, we further evaluated the antioxidant capacity of the *U. pertusa* ethanolic extract employing the DPPH radical scavenging method. This method is based on the reduction of an ethanolic DPPH solution in the presence of a hydrogen donating antioxidant thereby resulting in the formation of the non-radical form DPPH-H molecule upon the uptake of a hydrogen atom from antioxidant species (Schlesier et al. 2002). DPPH is a stable nitrogen centered free radical which can be effectively scavenged by antioxidants, hence, it has been widely used for rapid evaluation of antioxidant activity of plant extracts including seaweeds due to its reliability. The present study showed that the scavenging effect of the *U. pertusa* extract typically behaved in a concentration-dependent manner, i.e. the inhibitory effect increased with increasing concentration of the extract. Moreover, the IC_{50} value generated for *U. pertusa* ethanolic extract in the current study was lower compared with the *U. clathrata* extract but considerably higher than *U. linza*, *U. intestinalis*, and *U. flexuosa* methanolic extracts previously reported by Farasat et al. (2014). Several studies have by far elucidated the antioxidant activity in *Ulva* species. For example, among the 48 marine algae evaluated for their antioxidant activity, low antioxidant activity with a relatively high IC_{50} were documented for *U. intestinalis* (Zubia et al. 2007). However, some researchers have otherwise reported high free radical scavenging activity for other *Ulva* species. For instance, extracts of *U. compressa, U. linza,* and *U. tubulosa* exhibited high antioxidant activity in linoleic acid system, notably, with an excellent DPPH radical scavenging activity obtained for the methanolic extract of *U. compressa* as evidently supported by an IC_{50} of 1.89 mg mL^{-1} (Ganesan et al. 2011). Moreover, Qi et al. (2006) demonstrated that the natural ulvan, a group of sulfated heteropolysaccharides obtained from *Ulva* species, and its derivatives have higher scavenging activity on superoxide radical than vitamin C. Additionally, Hassan et al. (2011) accordingly documented that prolonged consumption of *U. lactuca* polysaccharides extract conferred potent antioxidant and hypocholesterolemic effects in experimentally-induced hypercholesterolemic rats. Corroborative to the published reports on *Ulva* species (Ganesan et al. 2011; Farasat et al. 2014), the antioxidant activity of *U. pertusa* extract in the current study likewise correlated with the quantity of its phenolic and flavonoid contents. As free radicals are formed during metabolism, i.e. as a result of the mitochondrial metabolism, inflammatory responses, phagocytosis, and physical activities, their production may be accelerated by external factors such as radiation, drugs and pesticides. As a consequence, overproduction of free radicals in the biological systems may damage all classes of chemicals including proteins, amino acids, nucleic acids, and carbohydrates in the biological materials (Sanjeewa et al. 2018). Thus, counteracting oxidative stress in the biological systems through the use of natural antioxidants such as those coming from seaweed extracts would be a practical strategy of protecting the organism from oxidative damage (Wang et al. 2010; Sanjeewa et al. 2018).

As shown in Table 3, *U. pertusa* ethanolic extract exhibited potent antibacterial activity against *S. aureus* ATCC25923. Li et al. (2018a) previously demonstrated that 10 mg mL^{-1} of the *U. pertusa* ethanolic extract

could induce a mean inhibition zone of 10.00±0.00 mm against *S. aureus*. Noticeably, such antibacterial activity is lower compared with the data that we generated in the present study since comparable values were markedly obtained when the *S. aureus* was exposed to a lower concentration (6.25 mg ml^{-1}) of the *U. pertusa* ethanolic extract. Our current findings consistently corroborate with the previous reports demonstrating the sensitivity of gram-positive *S. aureus* strains to seaweed extracts (Morales et al. 2006; Stirk et al. 2007), particularly against *U. pertusa* extract (Mtolera and Semesi 1996). *Staphylococcus aureus* typically constitutes the natural microbiota of the pond-reared fish and their culture environments (Pakingking et al. 2015). *Staphylococcus* species are generally not considered as fish pathogens as they have not by far been implicated in any serious disease outbreaks. However, among the principal foodborne bacteria described as human pathogens, *S. aureus* is the leading cause of gastroenteritis due to the consumption of fish products contaminated with this pathogen and its enterotoxin (Arfatahery et al. 2015). In fact, high population of these bacteria in harvested fish indicates the degree of spoilage it might have undergone. The extract likewise induced potent antibacterial activity against *S. agalactiae* isolates in a concentration-dependent manner. This finding is imperative as streptococcal infection among net-caged tilapias confined in open waters such as lakes have been recently recognized in the Philippines as a persistent problem that has resulted in serious economic losses (Legario et al. 2020). Fish farmers have resorted to using antibiotics, particularly amoxicillin (a newer version of penicillin), which is mixed with the feed (Legario et al. 2020). However, the emergence of multidrug-resistant *S. agalactiae* strains found in human and fish with high penicillin non-susceptibility has been recently reported (Li et al. 2020). Thus, the addition of *U. pertusa* extract in fish feed as an alternative prophylactic agent against streptococcal infection in cultured fish is worth pursuing. Of note, the capacity of *U. pertusa* ethanolic extract to inhibit the growth of *A. hydrophila* and *A. sobria* is an unequivocally important finding considering that these *Aeromonas* species are opportunistic pathogens which are ubiquitously found in fish and their rearing environments (Pakingking et al. 2015, 2020).

While there is no standard way to carry out a qualitative evaluation of the antimicrobial activity of a certain seaweed extract as different authors used different scales to assess in vitro antimicrobial susceptibility test (Silva et al. 2020), nevertheless, pertinent discrepancies observed with regard to variations in the antibacterial activity of the *U. pertusa* extract obtained in the current study and those previously reported for *U. pertusa* and other seaweed extracts feasibly lie on the extraction method employed, source and composition of macroalgae, and as well as protocols (method, time/ period/ season, etc.) utilized for the seaweed collection as previously reported (Stirk et al. 2007; Choi et al. 2014; Thanigaivel et al. 2016). For example, Choi et al. (2014) documented seasonal variations in the antimicrobial activity of the *U. pertusa* extracts against *Gardnerella vaginalis*; the extracts did not show activity during summer and autumn but notable activity from early winter (December) to middle spring (April). Despite the numerous studies by far conducted on the antimicrobial activities of the different seaweed extracts against fish pathogens, particularly those with zoonotic potential, there is still limited information on the exact mechanism of action for the majority of these extracts (Vatsos and Rebours 2015). Nevertheless, antibacterial compounds in seaweeds that have been considerably documented include fatty acids, lipophilic and phenolic compounds, lectins, acetogenins, terpenes, alkaloids, polyphenolics, isoprenoid metabolites, tannins, and hydrogen peroxide as reviewed by Mohamed et al. (2012). Thus, the potent antibacterial activity exhibited by the *U. pertusa* ethanolic extract against *S. aureus* in the current study could be attributed to these bioactive compounds present in the *U. pertusa* extract that we examined. Hitherto, pertinent investigations on *U. pertusa* ethanolic extract did not point out one individual substance or particular substance class which could be entirely responsible for its antimicrobial action, clearly indicating that a synergistic action is essential for the biological activity of the *U. pertusa* extract to be liberally expressed. Future studies should therefore isolate and characterize the major bioactive components of this green alga and their corresponding mode of action against important bacterial fish pathogens. Moreover, the practical therapeutic application of *U. pertusa* ethanolic extract against bacterial infections in tilapia and other susceptible high value marine fish species such as sea bass and groupers via feed supplementation is warranted.

Conclusion

In summary, our current data clearly demonstrated that *U. pertusa* ethanolic extract possesses potent antibacterial activity, particularly against infectious bacterial fish pathogens, and some bioactive components

with remarkable antioxidant capacities that may be utilized in the synthesis of novel drugs. With reference to the many studies by far conducted on the antimicrobial activities of the *U. pertusa* extracts that we reviewed, this study is the first to demonstrate the antioxidant and antibacterial activity of the ethanolic extract of *U. pertusa* commonly found in the Philippines and may therefore serve as promising alternatives for the prevention and control of infectious diseases in aquacultured species.

Authors' contributions The conception and design of the study were developed by RPJ and EGDJ-A. RPJ, DJL, RU, EGDJ-A, and CMC contributed to the data acquisition, analyses, and interpretation. RPJ wrote the manuscript with input from all authors. The final manuscript was approved by all authors.

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Conflicts of interest The authors declare that they have no conflict of interest.

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