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Bioactivity of Ellagic Acid and Velutin: Two Phenolic Compounds Isolated from Marine Algae

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MARINE macroalgae are considered as a promising source of chemical compounds with a broad array of biological activities. The bioactive compounds of five marine seaweeds (*Ulva lactuca, Padina boryana*, *Cystoseira myrica*, *Liagora farinosa* and *Acanthophora spicifera*) were extracted by chloroform, dichloromethane and ethanol. We managed to extract two polyphenolic compounds (ellagic acid and velutin) from the dichloromethane extract of *P. boryana* and ethanol extract of *A. spicifera*, respectively. The biological activities of the two isolated compounds as well as the crude extracts were screened. All tested algal extracts showed high antimicrobial activity against target bacteria and fungi except for *Pseudomonas aeruginosa* was resistant to *U. lactuca* and *C. myrica* extracts. Chloroform and ethanol extracts of *A. spicifera*, and dichloromethane extract of *C. myrica* showed the highest antimicrobial activity against *Bacillus subtilis* and *Staphylococcus aureus*. Dichloromethane extract of *P. boryana* had the highest antiprotozoal activity against *Trypanosoma cruzi* and *Leishmania donovani* (IC_{50} values, 3.5 and 4.8 µg/mL, respectively), and potent antioxidant activity up to 60 %. Overall, marine macroalgae with high polyphenols and flavonoids content exhibited excellent antimicrobial, antiprotozoal and antioxidant properties. Purified velutin recorded high antimicrobial activities as compared to the tested antibiotics and both ellagic acid and velutin also possessed considerable antiprotozoal activities. This study suggests that, ellagic acid and velutin comprise the key players for the antimicrobial and antiprotozoal activities of *P. boryana* and *A. spicifera* extracts, respectively. Thus, these two compounds could be used as pharmaceutically bioactive natural compounds.

Keywords: Antimicrobial, Antioxidant, Antiprotozoal, Ellagic acid, Marine algae, Velutin.

Introduction

Resistance to treatments by microorganisms enlarged in the previous years (Luepke et al., 2017), in addition to adverse effects on the host (Pradhan et al., 2016). Therefore, the development of antimicrobial drugs from natural sources that are greatly harmless, steadfast, and less costly is a requirement (Thanigaivel et al., 2015). Marine macroalgae are considered as a source of bioactive compounds as they yield excessive variability of secondary metabolites categorized by a wide range of biological activities (Pérez et al., 2016; Hamed et al., 2018). Recently, several researchers have paid attention to discover active compounds and extracts with antibacterial (Hellio et al., 2001; Kanimozhi & Sridhar, 2017), antifungal (Soares et al., 2016), antiprotozoal (Allmendinger et al., 2010; Spavieri

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et al., 2010) and antioxidant properties. These activities might be reflected in future applications in medicine, food production, or the cosmetic industry (Zubia et al., 2009; Stein et al., 2011). Fábio et al. (2014) summarized several useful products from macroalgae (e.g. fibers, minerals, vitamins, steroids, lectins, polyketides, mycosporine-like amino acids, proteins, polyphenols) and these products showed antioxidant prospective antibacterial, antifungal, antimalarial, anti-inflammatory, cytotoxic, anti-proliferative, anti-aging and anticancer activities (Sameeh et al., 2016; Ibraheem et al., 2017). Marine macroalgae also exhibited strong antiprotozoal and leishmanicidal activities with no cytotoxic effect towards mammalian skeletal myoblast cells (Orhan et al., 2006; Allmendinger et al., 2010; Spavieri et al., 2010) besides their great antioxidant potential (Kanimozhi & Sridhar, 2017). Phenolic compounds extracted from brown algae are considered to be a chemical defender against grazers and microbes (Plouguerne et al., 2006; Le Lann et al., 2008). Ellagic acid (EA) and velutin are two examples of highly active phenolic compounds. EA is commonly present in green tea and other natural sources including vegetables and fruits (Seeram et al., 2005; Fracassetti et al., 2013). It is a bioactive compound that has numerous prospective pharmacological and industrial uses (Sepúlveda et al., 2011). It acted as a potent natural antioxidant (Zhang et al., 2011), antitumor (Narayanan et al., 1999), antiviral activity (Ruibal et al., 2003) and antimicrobial agent (Ghudhaib et al., 2010). EA successfully repressed the manifestation of proinflammatory cytokines TNF-α and IL-6 in low micromole levels (Xie et al., 2012). Similarly, velutin showed cytotoxic activity and inhibits the human immunodeficiency virus (HIV-1) reverse transcriptase. It has been formerly isolated from winter mushroom, *Flammulina velutipes*, and açaí fruit pulp, *Euterpe oleracea* (Wang & Ng, 2001). Although, these two active compounds previously isolated from higher plants, it is the first time to be isolated from macro-algae with high content. The main goals of this work were designed to estimate the antimicrobial, antiprotozoal and antioxidant capabilities of the chosen marine algal species that were abundant along the coast of Red Sea, Safaga province, Egypt. Also, to detect the bioactive compounds in the potent extracts and studying their biological activities.

Materials and Methods

Algal material collection

Ulva lactuca Linnaeus (Chlorophyta), *Padina boryana* Thivy, *Cystoseira myrica* Agardh (Phaeophyta), *Liagora farinosa* Lamouroux and *Acanthophora spicifera* Vahl (Rhodophyta) were gathered from Safaga province at the Red Sea eastern coast, Egypt (26° 44' N and 33° 56' E). The algal specimens were washed by tap water to remove any epiphytes and associated debris then were cleaned using a brush with 5 % ethanol to remove the adhering microflora. The algal specimens were air-dried under shade at room temperature and were ground thoroughly by electrical blender and sieved through a 0.5 mm^2 sieve plate.

Preparation of algal extracts

The extractions were achieved according to the method designated by Hellio et al. (2001). The desiccated algal powders were soaked in chloroform, dichloromethane, and 95% ethanol, separately, at room temperature for 72hrs, every 500gm for 3L from each solvent. These algal solutions were then filtered through Whatman filter paper No.1. The obtained filtrates were concentrated under reduced pressure in the rotatory evaporator (GG SENCO) for complete dryness. The dried crude extracts were stored at 4°C for testing their antimicrobial activities.

Antimicrobial activity test

In the current study, the authors tested the antimicrobial activities of 15 algal extracts from the experimental marine macroalgal species and the two isolated polyphenolic compounds (ellagic acid and velutin) against gram-positive bacteria i.e., *Sarcina lutea (*ATCC 10773), *Staphylococcus aureus* (ATCC 25923) and *Bacillus subtilis* (A TCC 31324), gram-negative bacteria i.e., *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 12453). Antimicrobial activity was examined by the agar diffusion method as designated in European Pharmacopoe (1997). Mueller-Hinton medium was used for bacterial species cultivation, while, Sabouraud dextrose agar medium was used for *C. albicans* propagation. A swap from each solid broth of the respective microorganism suspended in 3 mL 0.85% sterile NaCl solution, individually. Saline suspension was carefully mixed with sterilized agar media after cooling down to 42^0C. A suitable volume of this seeded agar was dispensed individually into 9cm sterile Petri dishes and indorsed to harden. According to the preliminary test of minimal inhibitory concentration, 10µl of dimethyl sulfo-oxide (DMSO) was impregnated with 3mg of the respective algal extractor, 0.1mg of velutin, and ellagic acid that loaded onto a sterilized

paper disc (6mm diameter). The impregnated discs were sited separately from each other on the inoculated agar plate aseptically. DMSO-loaded discs were used as negative control, whereas, ampicillin (AM, 10µg/100µl), gentamicin (GM, 10mcg), and tetracycline (TE, 30mcg) antibiotic discs were used as a positive control. Cultures were kept for 3hrs at 10°C pre-diffusion before incubation (Bansemir et al., 2006). Then all of these cultures were incubated at 37°C and 28°C for the bacterial and fungus species, respectively. Clear zone size was measured in mm after 24hrs incubation, all measurements were conducted in triplicates.

Bioassay of antiprotozoal activity

In vitro antileishmanial examination against the amastigotes of *Trypanosoma cruzi* and *Leishmania donovani* was accompanied according to protocols of Räz et al. (1997). *Trypanosoma cruzi* (TIB2342) and *Leishmania donovani* (TIB5437) were obtained from Tropic institute, Belgium. Amastigote density was adjusted to 1×10^8 parasites per mL, and 90µL was supplementary to each well of 96-well plates. 10µL of each algal extract, ellagic acid, velutin or standard compounds e.g., Benznidazole, Miltefosine (Sigma Aldrich, USA) solutions were added to obtain the chosen concentrations. Amastigotes of *L. donovani* were incubated for 72hrs with Alamar Blue™, the viability in the microtitre plate was evaluated through color reaction detection by fluorescence scanner. Trypomastigotes viability measurement of *Trypanosoma cruzi* (Tulahuen C4) was piloted using rat skeletal myoblasts (L-6 cells) which was seeded in the microtiter plates exhausting the substrate chlorophenol red-β-D-galactopyranoside (CPRG)-Nonidet (Sigma Aldrich, USA). The microtiter plates were incubated for 4 days under a 5% CO_2 condition at 37°C and the developed color reaction during the first 24 h was read photometrically at 540nm. IC_{50} was evaluated for both species and each extract was tested in triplicate.

DPPH radical scavenging antioxidant activity

Algal extracts, ellagic acid, and velutin free radical scavenging activity were demonstrated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich, USA) method suggested by Hatano et al. (1988).

Total antioxidant capacity (FRAP)

Ferric reducing antioxidant power (FRAP) was evaluated by grinding 200mg of algal fresh wt. in 2mL 80% ice-cold ethanol in liquid nitrogen. FRAP reagent in the ratio of 10: 1: 1.180µL of newly set mixture was diluted with ethanol extracts and measured at 593nm by a microplate reader (Synergy Mx, Biotek Instruments Inc., Vermont, VT, USA) (Benzie & Strain, 1999). Trolox (Sigma Aldrich, USA) was used as a standard in the concentrations of zero to 650µM for calibration.

Estimation of total phenolic and flavonoids content

Total polyphenol and flavonoid content were extracted in 80% ethanol (v/v) and were estimated (Chang et al. 2002; Zhang et al., 2011; Casasole et al., 2017; Fina et al., 2017).

Isolation and identification of ellagic acid from P. boryana

Among the tested extracts, dichloromethane extract of *P. boryana* was selected as the most active extract. It possesses the highest antioxidant, antiprotozoal, and considerable antimicrobial activity. Therefore, it was retained for further purification steps and distributed sequentially to four fractions: F1 (n-hexane), F2 (acetone), F3 (n-butanol), and F4 (water). Acetone fraction (F2) showed the best results according to the antimicrobial bioassay test (Table 1). Depending upon the thin-layer chromatography (TLC) analysis, this fraction was fractionated on the silica gel column, using hexane: acetone (100:0) to (5:1) as mobile phase that yielded seven fractions (F2,1-F 2,7). The results showed that fraction no. F2,6 had the most inhibitory effect on antimicrobial growth. Further fractionation processes were conducted on a silica gel column using hexane: methanol (20:1 and then 10:1) which yielded three sub-fractions (F2,6,1-F2,6,3). Results of antimicrobial bioassay showed that sub-fraction no. F2, 6, and 3 had the highest activity. TLC analysis revealed that the sub-fraction was mainly a mixture of two active compounds.

In this study, the major active collected fraction was purified using high performance liquid chromatography (HPLC, Shimadzu class-LC 10 AD chromatograph supplied with Shimadzu SPD-10 AUV-VIS, phenomenex C18, 25cm × 4.6mm, 5µm particle size) to yield a pure compound. The purified compound was characterized by nuclear magnetic resonance analysis (NMR) based on spectroscopic analysis (^{1}H and ^{13}C NMR) (Fig. 1). Ellagic acid was equated with former studies values (Nduke et al., 2005; Srivastava et al., 2007).

TABLE 1. Antimicrobial bioassay activity of *Padina boryana* and *Acanthophora spicifera* dichloromethane fractions, as well as three commercial antibiotics against the tested microbes.

Alga	Fraction	Solvent	Inhibition zone (mm)				
			Bacillus subtilis	***Pseudomonas aeruginosa***	***Sarcina lutea***	***Staphylococcus aureus***	***Candida albicans***
Padina boryana	F1	n-hexane	8.9±0.4^{c}	11.9±0.7^{b}	12±0.5^{b}	18.9±1.2^{b}	18.9±1.1^{a}
	F2	acetone	22.2±1.2^{a}	19±0.9^{a}	24±1.1^{a}	22.9±1.1^{a}	18.4±0.9^{a}
	F3	n-butanol	10.7±1.3^{c}	5.6±0.8^{c}	11.5±1.0^{b}	23.3±2^{a}	12.3±1.5^{b}
	F4	water	15.5±0.8^{b}	10.7±1.6^{b}	26.7±0.7^{a}	19.1±1.6^{b}	17.7±2.8^{a}
Acanthophora spicifera	F1	$CHCl_3$–Ethanol gradients	26.0±0.6^{a}	17.3±1.0^{b}	21.2±0.1^{c}	20.7±1.5^{c}	8.3±0.6^{c}
	F2		20.0±0.6^{b}	0.0^{d}	28±0.4^{a}	18.9±1.7^{a}	12.4±1.2^{b}
	F3		21.0±1.3^{b}	11±1.5^{c}	25±3.1^{b}	23.9±1.3^{b}	17.3±0.9^{a}
	F4		26.1±0.9^{a}	20.0±0.5^{a}	31.3±1.2^{a}	25.3±2.1^{b}	18.3±1.5^{a}
	F5		16.9±1.0c	2.0^{d}	17.3±1.5^{d}	29.7±2.2^{a}	17±1.2^{a}
	F6		18±0.9^{b}	0.0^{d}	13.1±1.1^{e}	14.3±2.1^{d}	15.9±0.8^{b}
	F7		13.3±0.6^{d}	2.0±0.1^{a}	11.2±0.2^{f}	15.3±2.1^{d}	11.3±1.5^{c}
AM (10 µg/100 µl)			14.8±1.4^{de}	22.2±2.0^{d}	18.8±2.0^{e}	15.3±1.4^{d}	22.8±2.3^{e}
GM (10 mcg)			0.0^{a}	37.3±0.6^{h}	18.3±0.6^{d}	25.7±1.2^{e}	14.7±0.6^{c}
TE (30 mcg)			0.0^{a}	25.3±0.5^{a}	29.0±1.1^{a}	20.09±0.8^{a}	17.6±0.9^{a}
DMSO			0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}

- AM: Ampicillin, GM: Gentamicin, TE: Tetracycline and the used solvent DMSO (Dimethyl sulfo-oxide).
- Values are expressed as the mean of three biological replicates ± SD.
- Different letters represent significant differences between the treatments in the same raw. (Duncan test; P< 0.05; n= 3).

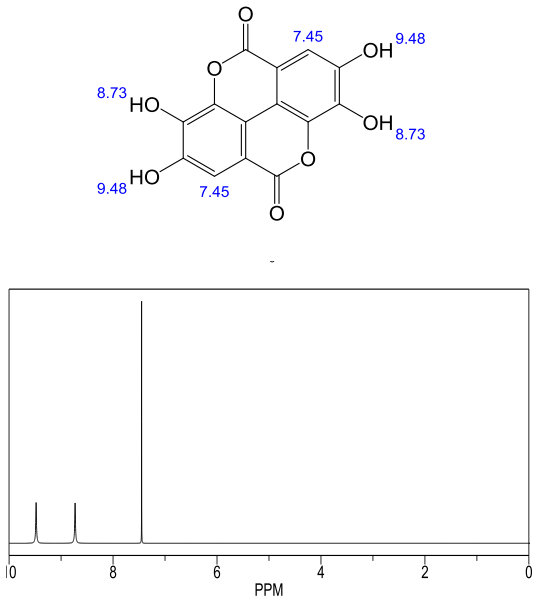

Fig. 1. ^{1}H-^{13}C NMR spectrum of the purified ellagic acid based on spectroscopic analysis.

Isolation and identification of velutin from A. spicifera

About 5kg of *A. spicifera* dry powder was extracted by 95% ethanol at room temperature as detailed earlier. The obtained filtrate was concentrated under reduced pressure in the rotatory evaporator till complete dryness to yield 710g of crude viscous material. Which was further partitioned with $CHCl_3$ to give a 69.5g yield. The $CHCl_3$-soluble fraction was fractionated in sephadex LH-20 column eluted by a gradient of $CHCl_3$–Ethanol to afford seven fractions (F1–7). Antimicrobial bioassay indicated that the highest active fraction was no. 4 (Table 1) which was further purified on silica gel column chromatography using 50 to 80% aqueous methanol giving up 13 sub-fractions (F4,1–F4,13). The F4,1 was left to crystallize at room temperature to yield 105.48 mg of yellow needles compound. The purified compound was characterized by nuclear magnetic resonance analysis (NMR) based on spectroscopic analysis (^{1}H and ^{13}C NMR) (Fig. 2).

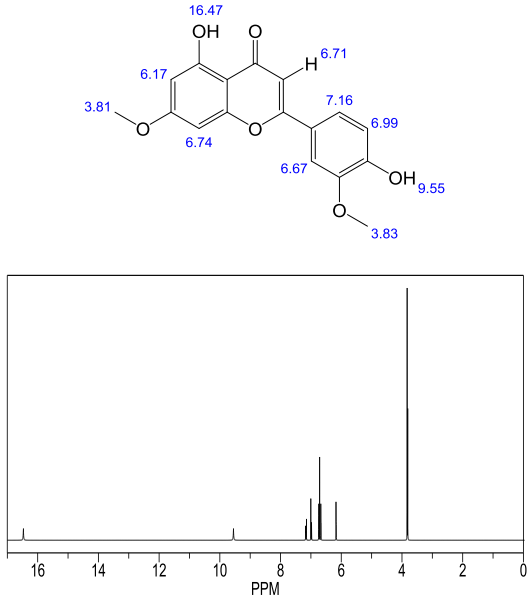

Fig. 2. ^{1}H-^{13}C NMR spectrum of the purified velutin based on spectroscopic analysis.

Statistical analysis

The data were analyzed by using SPSS (SPSS Inc, Chicago, Illinois, USA). One-way and two-way analyses of variance (ANOVA) were achieved on the original data to assess the impact of algal extract and isolated pure phenolic compounds. Significant differences between means were determined by using the Duncan test ($P< 0.05$).

Results

Antimicrobial activities

Fifteen different extracts from 5 marine algal species collected from Safaga coastline, Red Sea, Egypt were examined for their antimicrobial activity against five human pathogenic microbes (Table 2). All tested marine macroalgal extracts were active against the assayed bacteria and *C. albicans* except the three solvent extracts of *U. lactuca* and *C. myrica* which had no activity against *P. aeruginosa*. Similarly, tetracycline (reference antibiotic) showed no effect against all of the tested pathogens. Among the tested algae, *A. spicifera* and *P. boryana* extracts showed the highest antimicrobial activity but with variable levels which ranged between moderate, weak, and nil action. For instance, the chloroform extract of *A. spicifera* (3.0mg/disc) had the highest inhibitory effect against *B. subtilis* and *S. aureus* by 41 and 24.7 mm, respectively. Ethanol extract of *A. spicifera* also showed a relatively high antimicrobial activity against *B. subtilis*, *S. lutea* and *S. aureus* by 28.7, 28.1, and 24mm, respectively. Similarly, chloroform extracts of *P. boryana* (3.0mg/disc) exhibited a broad spectrum of antimicrobial activity against Gram (+) bacteria, *B. subtilis*, *S. lutea* by 26.5mm and *S. aureus* by 23.7 and Gram (-) bacteria, *P. aeruginosa* by 19.3mm.

Antiprotozoal activity

Generally, high polar extracting-solvent showed high antiprotozoal activity. For example, ethanol extract of *A. spicifera*, *C. myrica* and *L. farinosa* had higher antiprotozoal activity compared to other solvent extracts. Among the tested solvents dichloromethane extract of *P. boryana* showed the highest inhibition activity against *T. cruzi* and *L. donovani* with IC_{50} 3.5 and 4.8µg/mL respectively, followed by ellagic acid with IC_{50} 9.2 and 8.9µg/mL against *T. cruzi* and *L. donovani*, respectively (Table 3).

Antioxidant activities

The scavenging activity (DPPH) and total antioxidant capacity (FRAP) were dependent on algal species and the polarity of extracting a solvent. Where high polar extracting-solvent showed considerable antioxidant activity. Dichloromethane extract of *P. boryana* exhibited the highest DPPH and FRAP by 62.3 and 60.6%, respectively, as compared to the other macroalgal extracts which showed activity below ~ 50 %. In contrast, the chloroform extract of *P. boryana*, *C. myrica, L. farinose,* and *A. spicifera* showed the lowest DPPH scavenging activity and FRAP capacity (Table 4).

Total polyphenols content also varied among species from 0.02 to 0.9mg.g FW^{-1} (Table 5). The dichloromethane extract of *P. boryana* and ethanol extract of *A. spicifera* displayed the highest polyphenols content (0.9± 0.04mg.g FW^{-1}). Similarly, the highest flavonoids content was recorded for dichloromethane extract of *P. boryana* by 48.3 ± 6.7 µg.gFW^{-1} and ethanol extract of *A. spicifera* by 44.1 ± 2.6µg.gFW^{-1}. It is worthy to mention that chloroform extract recorded the lowest polyphenols and flavonoids contents among all tested solvents.

Identification of pure active phenolic compounds

The characteristic signals of ellagic acid using the ^{1}H NMR spectrum and ^{13}C-NMR spectrum (400MHz) were reported in our previous manuscript of Abu El-Soud et al. (2013) (Fig. 1). Meanwhile, velutin compound extracted in ethanol of *A. spicifera* was identified according

to ^{1}H-NMR (600 MHz, DMSO*d*6)^{TM} spectra: 4.07 (s, -OCH_3), 3.76 (s, -OCH_3), 6.63 (d. *J*= 2.4 Hz, H-6), 7.01 (d, *J*= 2.4 Hz, H-8), 6.93 (s, H-3), 6.94 (d, *J*= 8.4 Hz, H-52) 7.32 (brs, H-62), 7.45 (d, *J*= 1.8 Hz, H-22) (Fig. 2). Further comparison with previous data reported in Park et al. (2006) indicating that the purified compound was velutin.

Biological activity of ellagic acid and velutin

Interestingly the extracted polyphenolic compound velutin (0.1mg/disc) showed higher antibacterial activity against *P. aeruginosa* by 26.8 mm compared to ampicillin and other tested algal extracts (Table 2). Ellagic acid at 0.1mg/disc also showed comparable effect to other crude extracts which ranged between weak and moderate inhibition. Ellagic acid and velutin showed considerable activities against *T. cruzi* and *L. donovani* with a variable IC_{50} record, which ranged between 3.5µg/mL to > 90µg/mL (Table 3). The DPPH of ellagic acid and velutin were 21.4% and 20.4%, respectively, while FRAP was 30.1% and 23.6%, respectively (Table 4).

TABLE 2. Antimicrobial activity of algal extracts, the isolated polyphenolic compounds (ellagic acid and velutin) as well as three commercial antibiotics against the tested microbes.

Alga	Solvent	Inhibition zone (mm)				
		Bacillus subtilis	***Pseudomonas aeruginosa***	***Sarcina lutea***	***Staphylococcus aureus***	***Candida albicans***
Ulva lactuca (3mg/disc)	$CHCl_3$	6.3±0.6^{b}	0.0^{a}	8±0.2^{b}	16±1.7^{a}	11±1.1^{a}
	$MOHCl_2$	8.3±0.6^{bc}	0.0^{a}	8.7±0.6^{b}	15±0.8^{b}	7.3±0.6^{a}
	EOH	7.7±0.6^{b}	0.0^{a}	13.7±0.6^{c}	10±2^{b}	10.7±1.5^{a}
Padina boryana (3mg/disc)	$CHCl_3$	26.5±0.6^{j}	19.3±1.5^{d}	26.5±1.5^{e}	23.7±1.5^{a}	8.3±0.6^{b}
	$MOHCl_2$	20.0±0.6^{f}	12±1.5^{b}	28±0.4^{e}	19±1.7^{a}	12.4^{bc}
	EOH	21.0±1.3^{f}	11±1.5^{b}	25±1.2^{e}	23±1.4^{e}	17.3±1.2^{cd}
Cystoseira myrica (3mg/disc)	$CHCl_3$	11.3±0.6^{d}	0.0^{a}	11.3±1.2^{c}	25.3±2.1^{e}	13.3±1.5^{a}
	$MOHCl_2$	16±1.2^{e}	0.0^{a}	14.3±1.5^{c}	29±2.2^{f}	18±1.2^{d}
	EOH	13±0.9^{d}	0.0^{a}	13.7±1.5^{c}	18.3±2.1^{cd}	15±2.8^{c}
Liagora farinosa (3mg/disc)	$CHCl_3$	14.1±1.3^{e}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}
	$MOHCl_2$	15.3±1.4^{e}	12.5±1.1^{b}	18.6±1.8^{d}	15.9±1.4^{c}	7.6±0.7^{bc}
	EOH	17.8±1^{f}	11.7±1.1^{b}	27.1±2.52^{e}	10.4±1.0^{b}	11.1±1.1^{a}
Acanthophora spicifera (3mg/disc)	$CHCl_3$	41.1±3.7^{h}	15.7±1.4^{c}	0.0^{a}	24.7±2.2^{e}	8.6±0.8^{b}
	$MOHCl_2$	26.5±2.4^{j}	0.0^{a}	0.0^{a}	20.5±1.9^{e}	12.9±1.2^{b}
	EOH	28.7±2.6^{j}	0.0^{a}	28.1±2.6^{e}	24±2.2^{f}	18±1.6^{vd}
Ellagic acid (0.1mg/disc)		12.7±0.6^{d}	15.7±2.1^{c}	0.0^{a}	10.3±1.5^{b}	10±0.9^{b}
Velutin (0.1mg/disc)		13.2±1.2^{d}	26.8±2.4^{j}	22.4±2.2^{de}	10.7±1.0^{b}	10.4±0.9^{b}
AM (10µg/100 µl)		14.8±1.4^{de}	22.2±2.0^{d}	18.8±2.0^{d}	15.3±1.4^{c}	22.8±2.3^{e}
GM (10mcg)		0.0^{a}	37.3±0.6^{h}	18.3±0.6^{d}	25.7±1.2^{e}	14.7±0.6^{c}
TE (30mcg)		0.0^{a}	25.3±0.5^{a}	29.0±1.1^{a}	20.09±0.8^{a}	17.6±0.9^{a}
DMSO		0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}

- AM: Ampicillin, GM: Gentamicin, TE: Tetracycline and the used solvent DMSO (Dimethyl sulfo-oxide).
- Values are expressed as the mean of three biological replicates ± SD.
- Different letters represent significant differences between the treatments in the same raw. (Duncan test; P< 0.05; n= 3).

TABLE 3. Antiprotozoal activity, expressed as IC_{50} µg/mL, of algal extracts, the isolated polyphenolic compounds (ellagic acid and velutin), and the antiparasitic standards; Benznidazole[a] and Miltefosine[b] against *Trypanosoma cruzi* and *Leishmania donovani.* .

Test organism	Standard	*Ulva lactuca*			*Padina boryana*			*Cystoseira myrica*			*Liagora farinosa*			Acanthophora spicifera			Ellagic acid	Velutin
		$CHCl_3$	$MOHCl_2$	EOH	$CHCl_3$	$MOHCl_2$	EOH	$CHCl_3$	$MOHCl_2$	EOH	$CHCl_3$	$MOHCl_2$	EOH	$CHCl_3$	$MOHCl_2$	EOH		
T. cruzi	0.072± 0.0[a]	54.8± 1.5[f]	77.1± 3.2[j]	50.5± 2.4[f]	55.4± 4.1[f]	3.54± 0.1[b]	21.5± 2.3[e]	>90± 7.3[b]	>90 ±6.2[b]	62.5± 3.2[j]	>90 ±3.2[h]	>90 ±1[h]	52.9 ±1[f]	>90 ±3.6[h]	38.7 ±2.1[f]	15.98± 1[d]	9.2[a]± 0.87[c]	21.5 ±0.9[e]
L. donovani	0.205± 0.03[a]	48.6± 5.8[f]	65.87± 6.1[j]	37.9± 4.5[e]	31.78± 2.0[e]	4.87± 0.5[b]	19.0± 1[d]	>90± 5.2[h]	>90 ±3.7[b]	57.6± 2.8[j]	>90 ±2.9[h]	45.4 ±1.9[f]	27.45 ±2.2[de]	>90 ±7.0[h]	77.4 ±3.8[b]	24.11± 2.3[d]	8.87± 2.3[c]	29.5 ±2.1[d]

- Values are expressed as the mean of three biological replicates ± SD. Different letters represent significant differences between the treatments in the same raw. (Duncan test; P< 0.05; n= 3).

TABLE 4. Antioxidant capacity (DPPH and FRAP) of the algal extracts and the isolated polyphenolic compounds (ellagic acid and velutin). DPPH: 2,2-diphenyl-1-picrylhydrazyl, FRAP: ferric reducing antioxidant power.

Standard	*Ulva lactuca*			*Padina boryana*			*Cystoseira myrica*			*Liagora farinosa*			*Acanthophora spicifera*			Ellagic acid	Velutin
	$CHCl_3$	$MOHCl_2$	EOH	$CHCl_3$	$MOHCl_2$	EOH	$CHCl_3$	$MOHCl_2$	EOH	$CHCl_3$	$MOHCl_2$	EOH	$CHCl_3$	$MOHCl_2$	EOH		
DPPH (%)	27.9 ±1.6[d]	23.6 ±1.0[d]	45.1 ±4.0[f]	12.6 ±0.7[bc]	62.3 ±2.4[j]	42.8 ±2.8[ef]	5.3 ±0.82[a]	26.2 ±2.5[d]	16.1 ±2.2[c]	9.8 ±0.8[b]	24.1 ±3.9[d]	38.3 ±6.3[e]	12.7 ±1.2[bc]	34.0 ±4.0[f]	45.8 ±5.3[f]	21.4 ±0.2[f]	20.4 ±0.9[d]
FRAP (nmol /g FW)	26.9 ±3.8[c]	25.9 ±2.5[c]	46.9 ±1.1[e]	24.4 ±3.6[f]	60.6 ±2.3[f]	48.1 ±1.8[e]	5.5 ±1.4[a]	14.5 ±2.3[b]	12.7 ±1.3[b]	5.8 ±1.2[a]	14.4 ±0.7[f]	21.9 ±0.3[c]	14.3 ±1.6[b]	30.1 ±0.6[d]	37.3 ±2.2[d]	30.1 ±2.2[d]	23.6 ±1.4[c]

- Values are expressed as the mean of three biological replicates ± SD. Different letters represent significant differences between the treatments in the same raw. (Duncan test; P < 0.05; n=3)

TABLE 5. The concentration of total polyphenols and flavonoid in marine algal extracts.

Standard	*Ulva lactuca*			*Padina boryana*			*Cystoseira myrica*			*Liagora farinosa*			*Acanthophora spicifera*		
	$CHCl_3$	$MOHCl_2$	EOH	$CHCl_3$	$MOHCl_2$	EOH	$CHCl_3$	$MOHCl_2$	EOH	$CHCl_3$	$MOHCl_2$	EOH	$CHCl_3$	$MOHCl_2$	EOH
Polyphenol (mg/ g FW)	0.2 ±0.05[b]	0.6 ±0.02[d]	0.4 ±0.07[c]	0.1 ±0.01[b]	0.9 ±0.04[e]	0.4 ±0.0[c]	0.01 ±0.0[a]	0.4 ±0.13[c]	0.4 ±0.02[c]	0.02 ±0.01[a]	0.3 ±0.2[c]	0.4 ±0.03[c]	0.04 ±0.0[a]	0.4 ±0.15[c]	0.9 ±0.1[e]
Flavonoids (µg/ g FW)	5.4 ±1.2[b]	14.1 ±2.4[c]	16.5 ±3.5[c]	6.1 ±0.4[b]	48.3 ±6.7[j]	27.5 ±2.2[e]	1.9 ±0.3[a]	17.1 ±0.3[c]	12.9 ±1.9[d]	1.0 ±0.2[a]	17.2 ±1.6[d]	23.4 ±2.3[e]	5.3 ±2.0[b]	31.2 ±1.2[e]	44.1 ±2.6[f]

- Values are expressed as the mean of three biological replicates ± SD.
- Different letters represent significant differences between the treatments in the same raw. (Duncan test; P < 0.05; n=3)

Discussion

Marine macroalgae are an important renewable resource of bioactive compounds useful for healthy food and alternative medications capable of regulator diseases or multi- resistant strains of pathogenic microorganisms (Pérez et al., 2016). One of our main goals in this investigation is a quest for the gain of a new bioactive material from marine algal species. In the present study, all of the studied algal species had antimicrobial activity with a wide range of strength; weak, moderate, or strong effect against the tested microbes. Among fifteen different solvent extracts, chloroform and ethanol extract of *A. spicifera* and dichloromethane of *P. boryana* showed uppermost inhibitory effect against the tested pathogens as compared to other algal extracts. This finding was in a good correlation with the highest level of polyphenols and flavonoids in the dichloromethane extract of *P. boryana* and ethanol extract of *A. spicifera* which could explain their antimicrobial activities. In accordance, the antimicrobial activity correlates positively with the phenolic compounds content in the crude extracts of both *Nitraria retusa* and *P. boryana* that described by Mohamed et al. (2015) and Sameeh et al. (2016). Our results also revealed that the dichloromethane extract of *P. boryana* had considerable antiprotozoal and leishmanicidal activities against *T. cruzi* and *L. donovani* with IC_{50} 3.5 and 4.8µg/mL, respectively. Meanwhile, the obtained IC_{50} values were smaller than those reported by many investigators on the screening of crude extracts of green and brown macroalgae (Orhan et al., 2006; Spavieri et al., 2010; Süzgeç-Selçuk et al., 2011). However, *U. lactuta* and *P. oceanica* had the greatest leishmanicidal activity by IC_{50} 5.9 and 8.0 µg/mL, respectively (Orhan et al., 2006). In this context, methanolic extract of the brown alga, *Dasya pedicellata* showed antiprotozoal activity against *T. cruzi* trypomastigotes by IC_{50} 62.02µg/mL and leishmanicidal activity by IC_{50} 23.04µg/mL (Süzgeç-Selçuk et al., 2011). Our results revealed that the two extracted phenolic compounds; ellagic acid and velutin also showed considerable activities against *T. cruzi* and *L. donovani* with variable IC_{50} values which ranged between 9.2µg/mL – 29.5µg/mL compared to other algal extracts. This suggests that ellagic acid and velutin which constitute the principal proportion of polyphenolic compounds may be responsible for the antiprotozoal activity of both *P. boryana* and *A. spicifera*.

Marine algae are a rich source of bioactive secondary metabolites, including phenols and polyphenols (Andrade et al., 2013; Maharana et al., 2015; Fernando et al., 2016). In the present study, brown macroalgae had the highest antioxidant activity as described by Sameeh et al. (2016) and Tenorio-Rodriguez et al. (2017). Dichloromethane extract of *P. boryana* had the highest antioxidant activity among the other algal extracts. Accordingly, it could be potentially useful in the manufacturing of food for health promotion. A similar finding has been reported for *Eisenia arborea* (Tenorio-Rodriguez et al., 2017) and *Turbinaria ornate* (Kelman et al., 2012). We detected that all chloroform extracts recorded the lowest polyphenols and flavonoids contents as well as the lowest antioxidant activities compared to all tested solvents. This could assume the antioxidant properties of polyphenols and flavonoids compounds. Butsat & Siriamornpun (2016) and Mellouk et al. (2017) recognized the discrepancy in antioxidant activity to the solvent type and extraction time. They suggested a good correlation between total phenolic content and antioxidant activity depend on radical scavenging activities of DPPH, ABTS, and FRAP evaluation. Polyphenol are natural antioxidants found in plants such as ellagic acid and velutin. Yangthong et al. (2009) initiate an important difference in total phenolic content between the three macroalgal groups. Our results revealed that polyphenol content was fairly great in brown macroalga *P. boryana* compared with red and green macroalgae as formerly described (Wang et al., 2009). Several studies proposed extraordinary and effective antioxidant activities of polyphenols compound extracted from brown macroalgae (Zubia et al., 2009; Machu et al., 2015). Other abundant compounds in *P. boryana* were flavonoids, which possibly were co-extracted and might also support its antioxidant activity (Birasuren et al., 2013; Butsat & Siriamornpun, 2016; Kanimozhi & Sridhar, 2017; Mellouk et al., 2017). Based on the HPLC and NMR analyses, the extracted bioactive phenolic compounds were ellagic in the dichloromethane extract of *P. boryana* and velutin in ethanol extract of *A. spicifera*. These two phenolic compounds could explain the higher antimicrobial, antiprotozoal and antioxidant properties of these extracts. Consistently, many reports established that ellagic acid and velutin were powerful bioactive compound (Wang & Ng, 2001; Han et al., 2006; Ndukwe et al., 2008; Sepúlveda et al., 2011; Zhang et al., 2011; Xie et

al., 2012). Biological activity of ellagic acid was more effective than gentamycin and streptomycin (Ghudhaib et al., 2010).

Conclusion

Marine macroalgae contained high levels of hydrophilic bioactive compounds, such as polyphenols i.e. flavonoids, ellagic acid, and velutin. Due to high phenolic content, target marine macroalgae showed excellent antimicrobial, antiprotozoal and antioxidant properties. This study showed that dichloromethane, and ethanol solvents were effective for polyphenols extraction from *P. boryana* and *A. spicifera*. Dichloromethane extract of *P. boryana* showed the highest antiprotozoal and antioxidant activities. Results suggest that ellagic acid and velutin are the principal constituents of polyphenolic content may be responsible for the antimicrobial and antiprotozoal activity, which may serve as pharmaceutical bioactive natural compounds for possible application in food for health promotion.

Conflict of interests: The authors declare no conflict of interest.

Authors contribution: Sherif Hassan, Seham Hamed, and Hamada AbdElgawad planned and conceived the experiments of the presented idea. Sherif Hassan and Hamada AbdElgawad carried out and performed the experimental work. Mohammed Almuhayawi worked out the bioassay of antiprotozoal activity. Seham Hamed and Sherif Hassan wrote the first draft of the manuscript. All authors discussed the results and revised the manuscript. Sherif Hassan and Hamada AbdElgawad reply to the reviewers' comments and conducted with the journal's requirements till the paper finally published.

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النشاط الحيوي لحمض الإيلاجيك وفيلوتين: مركبان من الفينول معزولين من الطحالب البحرية

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تعتبر الطحالب البحرية مصدراً واعداً للمركبات الكيميائية مع مجموعة واسعة من الأنشطة البيولوجية. لعزل مركباتها النشطة حيوياً، تم إجراء عمليات استخلاص محتوياتها النشطة باستخدام الكلوروفورم ، ثنائي كلوروميثان والإيثانول لخمسة أنواع من الطحالب البحرية، وهم *Ulva lactuca* (الطحالب الخضراء) ، *Padina boryana* و *Cystoseira myrica* (الطحالب البنية)، و *Liagora farinosa* و *Acanthophora spicifera*. تمكنا من استخلاص اثنين من مركبات البوليفينول (حمض الإيلاجيك و فيلوتين) من مستخلص ثنائي كلوروميثان من *P. boryana* ومستخلص الإيثانول من *A. spicifera*، على التوالي. كما فحصنا الأنشطة البيولوجية للمركبين المعزولين وكذلك الاستخلاصات المختلفة. أظهرت النتائج أن جميع مستخلصات الطحالب المختبرة أظهرت نشاط عالي مضادًا للبكتيريا والفطريات المستهدفة باستثناء *Pseudomonas aeruginosa* وكان مقاومًا لمستخلصات *U. lactuca* و *C. myrica*. من بين المذيبات التي تم اختبارها، أظهرت مستخلصات الكلوروفورم والإيثانول لـ *A. spicifera*، ومستخلص ثنائي كلورو ميثان لـ *C. myrica* أعلى نشاط مضاد للميكروبات ضد بكتيريا *Bacillus subtilis* و *Staphylococcus aureus*. كان لمستخلص ثنائي كلوروميثان من *P. boryana* أعلى نشاط مضاد للبكتيريا ضد *Trypanosoma cruzi* و *Leishmania donovani* (قيم IC50، 3.5 و 4.8 ميكروغرام / مللي ، على التوالي) ، ونشاط مضاد للأكسدة قوي يصل إلى 60 %. بشكل عام ، أظهرت الطحالب محل الدراسة ذات المحتوى العالي من البوليفينول والفلافونويد خصائص ممتازة مضادة للميكروبات ومضادة للبكتيريا ومضادات الأكسدة. سجل فلوتين المعزول أنشطة مضادة للميكروبات عالية مقارنة بالمضادات الحيوية التي تم اختبارها، وكان لكل من حمض الإيلاجيك والفيلوتين أيضًا أنشطة مضادة للبكتريا كبيرة. اقترحت دراستنا أن حمض الإيلاجيك والفيلوتين هما المكونان الرئيسيان لمحتوى البوليفينول الذي يمكن أن يفسر الأنشطة المضادة للميكروبات ومضادات الميكروبات لمستخلصات الطحالب. يمكن استخدام هذين المركبين كمركبات طبيعية حيوية بيولوجية للاستخدام المحتمل في الغذاء من أجل التقدم الصحي