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In Vitro Antibacterial Activity of Flavonoid Extracts of Two Selected Libyan Algae against Multi-Drug Resistant Bacteria Isolated from Food Products

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Abstract

This study aimed to evaluate the antibacterial activity of flavonoids extracted from two Libyan brown algae namely *Cystoseira compressa* and *Padina pavonica* using microwave-assisted extraction method against pathogenic bacteria isolated from meat, meat products, milk and dairy products (*Staphylococcus aureus* subsp. *aureus* (5 isolates), *Bacillus cereus* (3 isolates), *Bacillus pumilus* (1 isolate), *Salmonella enterica* subsp. *enteric* (4 isolates) and Enterohaemorrhagic *Escherichia coli* O157 (EHEC O157) (4 isolates)). All of these isolates were muti-drug resistant with high MAR index. The results showed that *C. compressa* extract exhibited better and stronger antibacterial activities against the seventeen tested isolates with inhibition zones diameter ranged from 14 - 22 mm compared to *P. pavonica* extract which showed positive effect against 9 isolates with low inhibition zone ranged from 11 - 16.5 mm. Flavonoids extracted from *C. compressa* also displayed the best spectrum of bactericidal effect with a ratio MBC/MIC ≤ 4 obtained on all susceptible tested bacterial strains. Flavonoids and proanthocyanidins significantly contributed to the antibacterial properties. The mode of action of these active extracts is under investigation.

Keywords

Brown Algae, Flavonoids, Multi-Drug Resistant Bacteria, Antibacterial Activity

1. Introduction

Seaweeds contain various bioactive metabolites which can benefit human health [1] [2]. They are currently in different phases of clinical trials [3] [4] due to their highly content of terpenes, alkaloids as well as phenolic compounds. The Phenolic compounds in algae are less reported than that in higher plants [5].

Although flavonoids are the most important polyphenolic compounds, few reports have paid attention to flavonoids from marine sources. The flavonoids are members of a class of natural compounds that recently have been the subject of considerable scientific and therapeutic interest. Flavonoids are known to contain a broad spectrum of chemicals and biological activities including antioxidant and free radical scavenging properties, antibacterial, antiviral, anticancer, anti-inflammatory, anti-allergic and also as potential therapeutic agents against a wide variety of diseases [6] [7] [8]. However on the basis of earlier reports, presence of flavonoids remained questionable in marine algae [9]. Evidence of flavonoid has only been reported from *Acanthophora spicifera* (Vahl) Børgesen [10] [11]. In 2009 Sabina and Aliya [12] had isolated Scutellarein 4'-methyl ether from red algae which was showed several bioactivity including anti-allergic [13], anticancer and anti-cytotoxic [14] activities *in vitro* and *in vivo*. Several extraction techniques and solvents are used for obtaining antioxidant and antibacterial agents from natural sources. Extraction techniques include solvent extraction (SE), soxhlet extraction, ultrasonication assisted extraction and supercritical fluid extraction [15] [16] [17]. Moreover, microwave assisted extraction (MAE), a new extraction method, has been introduced, in order to reduce the economical, environmental and durational costs of the extraction as well as to improve extraction yield [18] [19].

The importance of microbes to food products of animal origin had been demonstrated by recent outbreaks of food-borne illness associated with consumption of meat, milk and dairy products that had been contaminated with pathogenic organisms or toxins. Undesirable microorganisms constitute the primary hazard to safety, quality, and wholesomeness of food products. Recently there is urgent need to find new antibacterial agents due to the wide spread of drug-resistant bacteria [20]. These drug-resistant bacteria are increasing due to the resistivity that bacteria have developed and lack of new antimicrobials to combat them. Seaweeds have been proven to be a good source of antibacterial agents [21] [22] [23] [24].

As plants synthesized flavonoids as a response action to microbial infection; therefore it is expected that flavonoids are a good antimicrobial agent against various microorganisms. The antibacterial activity of flavonoids depends on the structures, namely on the substitutions on the aromatic rings. Antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesion, enzymes, cell envelope

transport proteins, and so forth. Lipophilic flavonoids may also disrupt microbial membranes [25] [26].

The objective of this study was to assess the antibacterial activity of flavonoids extracted from two Libyan brown algae using microwave-assisted extraction method against multi-drug resistant bacterial strains.

2. Materials and Methods

2.1. Collection and Processing of Algal Samples

Cystoseira compressa (*C. compressa*) and *Padania pavonica* (*P. pavonica*) were collected from western coast of Libya (SA1, N32 53.764 E13 20.990, SA 02, N32 53.756 E13 21.064; SA 03, N32 53.792 E13 21.070; SA 04, N32 53.804 E13 21.028; SA 05, N32 53.777 E13 20.983) between June and August 2015 (Figure 1). The algal samples were taxonomically identified at Marine plankton and algae department, Marine biology research center, Tajura-(east of Tripoli), Libya. Algae samples were cleaned by removing the epiphytes and necrotic parts. Samples were rinsed with sterile water and shade dried for 7 - 14 days and ground thoroughly to powder in a kitchen-type blender.

2.2. Preliminary Phytochmical Tests

Preliminary phytochmical tests for identification of alkaloids, anthraquinones, coumarins, flavonoids, saponins, tannins, and terpenes were carried out for all the extracts using standard qualitative methods that have been described previously [27].

2.3. Microwave Assisted Extraction (MAE) of Flavonoid

Experiments were carried out in a domestic (Black & Decker, Model No. MZ

Figure 1. Localization of the collection site of algae.

3000 PG, SL13YD, England) microwave oven system. Twenty five grams of the powdered plant materials were mixed with solvents (ethanol or methanol 80%) at a suitable ratio (500 ml). An intermittent microwave irradiation method was used to keep the temperature of the extraction mixtures below 80˚C [28]. The suspension was radiated in microwave oven at regular intervals (30 sec radiation and 30 sec off). Variation in irradiation time from one algae to another was dependent on the result of an assay-guided purification *i.e.* a quick flavonoids TLC spot test on the extraction products, using mobile phase 9:1 Benzene: Methanol. Extraction was stopped when the spot test indicted maximum yield for the tested algae (data not shown). The infusions were allowed to cool down to room temperature, filtrated and stored at (4˚C) for further analysis.

2.4. Antibacterial Activity Assay

Antibacterial activity assay was accomplished in the Department of Microbiology and Parasitology, Faculty of Veterinary Medicine; and Biochemistry Lab, Chemistry Department, Faculty of Science, University of Tripoli, Libya.

2. 4.1. Isolation and Identification of Bacterial Isolates

Standard microbiological methods [29] [30] were used to isolate 8 Gram negative (S. *enterica* 17 (Sal 1), *S. enterica* 18 (Sal 2), *S. enterica* 19 (Sal 3), *S. enterica* 29 (Sal 4), EHEC O157 57 (E1), EHEC O157 55 (E2), EHEC O157 52 (E3), EHEC O157 *49* (E4)) and, 9 Gram positive isolates (*S. aureus* 122 (S1), *S. aureus* 128 (S2), *S. aureus* 287 (S3), *S. aureus* 125 (S4); *S aureus* 283 (S5), *B. cereus* 4 (B1), *B. cereus* 16 (B2), *B. cereus* 72 (B3), *B. pumilus* 124 (B4)) from meat, meat products, milk and dairy products collected from different parts of Libya. All of the tested isolates were identified and characterized by culturing in the specific appropriate media followed by using conventional biochemical tests as well as partial sequencing of 16S rDNA as described by Azwai *et al*., 2016 [31].

2.4.2. Standardization of Bacterial Suspension

The bacterial suspensions were standardized following the CLSI guidelines for aerobic bacteria [32]. All of the tested bacteria were grown in Mueller Hinton Broth (MHB, Hi-Media) for 18 - 24 h, followed by the matching of bacterial suspension to the turbidity equivalent to 0.5 McFarland solution (1.5×10^8 CFU/mL) with the addition of sterile saline.

2.4.3. Antibiotic Sensitivity Test

Susceptibility against antimicrobials was performed against all of the above mentioned isolated bacterial strains by disk diffusion method according to standard microbiological protocol (National Committee for Clinical Laboratory Standards) [33] on Mueller-Hinton agar (Oxoid) using 24 of commonly used antibiotics; Amoxycillin (10 µg, AML), Amoxycillin/clavulanic acid (30 µg, AM-C), Ampicillin (10 µg, AMP), Bacitracin (10 µg, B), Penicillin (10 µg, P), Methicillin (5 µg, ME), Erythromycin (15 µg, E), Gentamycin (10 µg, CN), Kanmycin (30 µg, K), Lincomycin (10 µg, MY), Tobramycin (10 µg, TOB), Vancomycin (30 µg, VA),

Levofloxacin (5 µg, LEV), Clindamycin (DA), Cefotaime (30 µg, CTX), Doxycycline (30 µg, DO), Ciprofloxacin (5 µg, CIP), Cloxacillin (O B), Nitrofurantoin (F), Oxytetracyclin (30 µg, OT), Streptomycin (30 µg, S), Tetracycline (300 µg, TE), Chloramphenicol (30 µg, C), and Sulphamethoxazole/Tri-methoprim (25 µg, SXT). Interpretation of the results namely sensitive (S), intermediary resistant (I) and resistant (R) were made in accordance to the standard measurement of inhibitory zones in millimeter (mm).

2.4.4. Multiple Antibiotic Resistances (MAR) and Inhibition Resistance Index (ARI) of Test Isolates

The antibiotic susceptibility patterns obtained from the standard disc diffusion procedure were used to calculate the MAR and ARI index for total number of isolates as follow:

$$\text{ARI} = y/nx$$

where, y is the number of resistant isolates, n is the number of isolates and x is the number of antibiotics [34] and

$$\text{MAR index} = a/b$$

where, a is the number of antibiotics to which the isolates are resistant and b is the total number of antibiotics exposed. A MAR value ≥0.2 indicates that antibiotics are ineffective.

2.4.5. Antimicrobial Assay

The antimicrobial activity of algal flavonoid extracts was performed *in vitro* using the "hole-plate diffusion method" [35] at a concentration of 2000 µg/ml. Each test isolate strain was maintained in cryocare bacterial preservation (cryobeads) stored at −70˚C and was recovered for testing by growth in Mueller-Hinton (M-H) broth (Oxiod, England) for 24 h at 37˚C before testing. The inoculums suspension contains approximately 1.5×10^8 CFU/mL of bacteria. Each extract was placed into wells of 8 mm diameter. The plates were kept for 1h at 4˚C for allowing better diffusion of the extract into the agar. Subsequently, plates were incubated at 37˚C for 18h. Methanol was used as a negative control. Diameters of inhibition zones (DIZ) were measured in mm and the results were recorded as the mean of triplicate experiments.

2.4.6. Determination of Minimum Inhibitory Concentration (MIC)

The MIC was defined as the lowest concentration that completely inhibited the growth for 24 h. The MIC for the flavonoids extracts was determined by the macrodilution agar method. In the macrodilution agar method, a two-fold serial dilution of the flavonoids extracts was prepared in sterile freshly prepared Mueller-Hinton (M-H) broth used as diluents to achieve a decreasing concentration ranging from 500 to 20 µg/ml. Sterile cork borer of 8.0 mm diameter was used to bore well in the pre-solidified Mueller-Hinton (M-H) plates and 150 µl volume of each dilution was added aseptically into the wells made in M-H plates in triplicate that had bacteria seeded with the standardized inoculum (1.5×10^8 CFU/

ml). All the test plates were incubated at 37˚C and were observed for the growth after 24 hr [36].

2.4.7. Determination of Minimum Bactericidal Concentration (MBC)

The content of the MIC tubes and the content of the preceding tubes in the serial dilutions (2MIC, 3MIC and 4MIC) were subcultured into the MH broth. All bacterial plates were inoculated at 37˚C for 24 hours after which they were examined. MBC was the lowest concentration that completely inhibited bacterial growth. To confirm the results of MBC, 1 ml of the experimental suspensions was resubcultured in the M-H broth which were incubated at 37˚C for 18 - 24 h.

2.5. Determination of Total Flavonoid Content

Total flavonoid content was estimated according to [37]. 1 ml of algal extract (0.1 g/ml) was diluted with 4 ml of water and was mixed with 0.3 ml of $NaNO_2$ (5% w/v). After 5 min, 0.3 ml of $AlCl_3$ (10% w/v) was added followed by the addition of 2 ml of NaOH (1 M) six minutes later. The reaction volume was increased up to 10 ml by adding 2.4 ml distilled water and the sample was incubated at room temperature (RT) for 15 min. The absorbance was measured at 510 nm using spectrophotometer (Jenway Model 6405). The assay was performed in triplicate, and the flavonoids content was determined by interpolating the absorbance of the samples against a calibration curve constructed with rutin standard (1.25 to 20 mg/ml) and expressed as milligrams of rutin equivalent per gram of extract (mg RE/g).

2.6. Determination of Proanthocyanidin Content

Total proanthocyanidin content was determined according to Li's method [38]; 0.5 mL of the flavonoids extracts (4 mg/ml) was added to 3 mL of 4% (w/v) vanillin in methanol and 1.5 mL of 35% HCl and then incubated at RT for 15 min in dark place. After which, absorbance was recorded at 500 nm by UV/visible spectrophotometer. Results were expressed as mg catechin equivalents per gram (mg CE/g of the extract) for each sample.

2.7. Statistical Analysis

Data were expressed as means ± standard deviations (SD) of triplicate determinations. All statistical analyses were carried out using SPSS V.16 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL). Statistical differences between extract activities were determined using ANOVA followed by Least Significant Difference (LSD) testing. Differences were considered statistically significant when $P < 0.05$. The Pearsons correlation analysis was performed between antibacterial activity, total flavonoids and proanthocyanidin contents.

3. Results

Phytochemical screening of *C. compressa* and *P. pavonica* extracts showed the presence of most important phytoconstituents (results not shown). Flavanoids

were presented in noticeable amounts in both *C. compressa* and *P. pavonica*.

The high content of flavonoids in the investigated algae, drawn our interest toward application of the recently adopted microwave procedure for the efficient extraction of these phytochemicals.

3.1. Antibiotic Sensitivity Test of Bacteria

A total of 17 isolates were obtained from milk, dairy products, meat and meat products. Based on standard microbiological techniques and 16S rDNA gene sequence, the isolates were identified as *Staphylococcus aureus subsp. aureus* (5 isolates), *Bacillus cereus* (3 isolates), *Bacillus pumilus* (1 isolates), *Salmonella enterica subsp. enteric* (4 isolates) and Enterohaemorrhagic *Escherichia coli* O157 (EHEC O157) (4 isolates) [31].

The 16S rDNA gene sequences of isolates are deposited at Libyan GenBank under accession numbers *as S. aureus* 122 (S1), *S. aureus* 128 (S2), *S. aureus* 287 (S3), *S. aureus* 125 (S4); *S aureus* 283 (S5), *B. cereus* 4 (B1), *B. cereus* 16 (B2), *B. cereus 72* (B3), *B. pumilus* 124 (B4), S. *enterica* 17 (Sal 1), S. *enterica* 18 (Sal 2), S. *enterica* 19 (Sal 3), S. *enterica* 29 (Sal 4), EHEC O157 57 (E1), EHEC O157 55 (E2), EHEC O157 52 (E3), EHEC O157 49 (E4).

Antibiotic Sensitivity Patterns of Pathogenic Isolates

Susceptibility tests were conducted with 24 antibiotics against the tested isolates. The antibiotic profile for each pathogenic bacterium was determined using 24 commercial antibiotic discs (Table 1 and Table 2). Over all the isolates were resistant to at least 7 different antibiotics, showing that all isolates were multi-drug resistant. *S. enterica* isolates: *S. enterica* 17; *S. enterica* 18; *S. enterica* 19; *S. enterica* 29 were resistant to 14, 22, 13 and 10 antibiotics used respectively, EHEC O157 isolates: EHEC O157 57 and EHE O157 52 were resistance to 13 antibiotics while EHEC O157 55 and EHEC O157 49 were resistant to 18 and 15 antibiotics respectively. Among 9 Gram positive isolates, *S. aureus*: *S. aureus* 122; *S. aureus* 128, and *S. aureus* 125 were resistant to 5, 11, 10 respectively while *S aureus* 283 and *S. aureus* 287 were resistance to 12 antibiotics, results showed that all *S. aureus* tested were methicillin resistant *S. aureus* (MRSA) and *Bacillus cereus*: *B. cereus* 4, *B. cereus 16*; and *B. cereus 72* and *B. pumilus* 124 were resistance to 10, 9, 18 and 7 antibiotics respectively (Table 1 and Table 2).

The MAR index is the ratio of number of antibiotics ineffective against the organisms to the total number of antibiotics used [39]. The MAR index values of the tested organisms were reported in Table 3 and Table 4. The MAR index of isolated bacteria was greater than 0.2, which indicates that most tested antibiotics are ineffective. The results indicated that Gram negative isolates showed significantly greater resistance and higher MAR indices (0.63 - 0.96) than Gram positive isolates, with MAR index ranged from 0.38 - 0.83. In addition, the highest ARI value 0.19 was calculated for *S. enterica*, followed by 0.18 and 0.15 for EHEC O157 and *B. cereus*. In comparison, the lowest ARI value 0.11 was obtained by *S. aureus* (Table 1 and Table 2).

Table 1. Antibiotic susceptibility results of multidrug resistant Gram-positive bacteria.

Antibiotics	Bacterium								
	B. cereus and B. pumilus				*S. aureus*				
	B1	B2	B3	B4	S1	S2	S3	S4	S5
AML	R	R	R	R	R	R	S	R	S
AMC	R	R	R	S	S	S	S	S	S
AMP	R	R	R	R	R	S	S	R	S
B	R	R	R	R	I	S	S	I	S
P	R	R	R	R	R	I	S	R	S
ME	R	R	R	S	R	R	R	R	R
E	I	I	R	S	I	R	R	I	R
CN	S	S	R	S	S	S	R	S	R
K	S	I	R	S	S	S	R	S	R
MY	R	R	R	R	S	I	R	S	R
TOB	S	S	R	S	S	S	R	S	R
VA	S	S	I	S	S	S	I	I	I
LEV	S	S	S	S	S	S	S	I	S
DA	I	I	R	R	S	R	R	R	R
CTX	R	R	R	R	R	R	R	R	R
DO	S	S	I	S	S	S	R	S	R
CIP	I	S	S	S	I	S	S	I	S
OB	R	R	R	I	I	R	R	R	R
F	S	S	S	S	I	R	S	R	S
OT	I	I	R	S	S	R	R	R	R
S	S	I	R	I	S	R	S	I	S
TE	S	S	R	S	S	R	R	S	R
C	S	I	S	S	S	R	S	R	S
SXT	R	I	R	S	S	R	S	R	S
MAR Index	0.58	0.67	0.83	0.38	0.41	0.58	0.54	0.71	0.58
ARI	0.08				0.03				

R: Resistant; S: Sensitive; I: Intermediate; MAR index: Multi drug resistance; Antibiotics (μg/disc), AML: Amoxycillin 10; AMC: Amoxycillin/clavulanic acid 30; AMP: Ampicillin 10; B: Bacitracin 10; P: Penicillin 10; ME: Methicillin 5; E: Erythromycin 15; CN: Gentamycin 10; K: Kanmycin 30; MY: Lincomycin 10; TOB: Tobramycin 10; VA: Vancomycin 30; LEV: Levofloxacin 5; DA: Clindamycin 2; CTX: Cefotaime 30; DO: Doxycycline 30; CIP: Ciprofloxacin 5; OB: Cloxacillin 5; F: Nitrofurantoin 300; OT: Oxytetracyclin 30; S: Streptomycin 10; TE: Tetracycline 30; C: Chloramphenicol 30; SXT: Sulphamethoxazole/Trimethoprim 25.

Table 2. Antibiotic susceptibility results of multidrug resistant Gram-negative bacteria.

Antibiotics	Bacterium							
	*EHEC O*157				*S. enterica*			
	E1	E2	E3	E4	Sal 1	Sal 2	Sal 3	Sal 4
AML	R	R	R	S	R	R	R	I
AMC	S	S	S	R	S	R	I	I
AMP	R	R	R	R	S	R	I	S
B	R	R	R	R	R	R	R	R
P	R	R	R	R	R	R	R	I
ME	S	R	R	R	R	R	R	I
E	R	R	R	R	R	R	R	R
CN	R	S	R	S	I	R	S	S
K	I	S	I	I	I	R	I	S
MY	R	R	R	R	R	R	R	R
TOB	R	I	R	I	R	R	S	S
VA	R	R	R	R	R	R	R	R
LEV	S	S	S	R	S	R	S	S
DA	R	R	R	R	R	R	R	R
CTX	I	I	I	I	S	I	I	I
DO	R	S	R	S	R	R	R	R
CIP	S	S	R	R	S	R	S	S
OB	R	R	R	R	R	R	R	R
F	I	S	I	S	S	S	I	S
OT	R	R	R	R	R	R	R	R
S	R	R	R	S	R	R	R	R
TE	R	R	R	S	R	R	R	R
C	S	S	S	S	S	R	S	S
SXT	S	S	R	S	S	R	S	S
IndexRAM	0.75	0.63	0.88	0.67	0.63	0.75	0.96	0.67
ARI	0.11				0.14			

R: Resistant; S: Sensitive; I: Intermediate; MAR index: Multi drug resistance; Antibiotics (µg/disc), AML: Amoxycillin 10; AMC: Amoxycillin/clavulanic acid 30; AMP: Ampicillin 10; B: Bacitracin 10; P: Penicillin 10; ME: Methicillin 5; E: Erythromycin 15; CN: Gentamycin 10; K: Kanmycin 30; MY: Lincomycin 10; TOB: Tobramycin 10; VA: Vancomycin 30; LEV: Levofloxacin 5; DA: Clindamycin 2; CTX: Cefotaime 30; DO: Doxycycline 30; CIP: Ciprofloxacin 5; OB: Cloxacillin 5; F: Nitrofurantoin 300; OT: Oxytetracyclin 30; S: Streptomycin 10; TE: Tetracycline 30; C: Chloramphenicol 30; SXT: Sulphamethoxazole/Trimethoprim 25.

(Table 3 and Table 4) represent the antibacterial screening of the flavonoids extracted by MW, on Gram negative and Gram positive isolates. The results showed that *C. compressa* extract exhibited better and stronger antibacterial activities against 14 tested isolates with inhibition zones diameter ranged from 14 -

Table 3. *In vitro* antimicrobial activity of the algal flavonoids extracts against Gram positive bacteria isolated from food products. *S. aureus* (5 isolates), *B. cereus* (3 isolates), and *B. pumilus* (1 isolate).

	C. compressa	*P. pavonica*	
	DIZ (mm)	DIZ (mm)	MAR Index
	***S. aureus* isolates**		
S1	**18.5 ± 1.5^{c}**	13.5 ± 1.5^{b}	0
S2	**14 ± 1^{d}**	11.5 ± 0.5^{c}	0
S3	**20.5± 0.5^{b}**	11.5 ± 0.5^{c}	0
S4	-	-	1
S5	-	-	**1**
ARI		**0.41**	
	***B. cereus* isolates**		
B1	22 ± 2^{a}	-	0.5
B2	20.5 ± 0.5^{b}	16.5 ± 0.5^{a}	0
B3	17.5 ± 0.5^{c}	-	0.5
B4	-	-	1
ARI		**0.5**	

S1: *S. aureus* 122; S2: *S. aureus* 128; S3: *S. aureus* 287; S4: *S. aureus* 125; S5: *S aureus* 283; B1*: B. cereus* 4; B2: *B. cereus* 16; B3: *B. cereus* 72; B4: *B. pumilus* 124. Different letters indicate statistically significant differences between groups ($P < 0.05$).

Table 4. *In vitro* antimicrobial activity of the algal flavonoids extracts against Gram negative bacteria isolated from food products. *S. enterica* (4 isolates) and EHEC O157 (4 isolates).

	C. compressa	*P. pavonica*	
Bacterium	DIZ (mm)	DIZ (mm)	MAR Index
	S. *enterica isolates*		
Sal 1	31 ± 1^{a}	27.5 ± 0.5^{a}	0
Sal 2	17.5 ± 0.5^{c}	-	0.5
Sal 3	17 ± 1^{c}	-	0.5
Sal 4	24.5 ± 0.5^{b}	14.5 ± 0.5^{b}	0
ARI	**0.5**		
	EHEC O157 *isolates*		
E1	17 ± 0^{c}	-	0.5
E2	16.5 ± 1.5^{c}	-	0.5
E3	15 ± 0^{d}	-	0.5
E4	16 ± 0^{c}	-	0.5
ARI	**0.5**		

Data are expressed as the mean ± standard deviation (SD) of three replicates. Sal 1: *S. enterica* 17; Sal 2: *S. enterica* 18; Sal 3: *S. enterica* 19; Sal 4: *S. enterica* 29; E1*:* EHEC O157 57; E2: EHEC O157 55; E3: EHEC O157 52; E4: EHEC O157 49. Different letters indicate statistically significant differences between groups ($P < 0.05$).

22 mm compared to *P. pavonica* extract which was observed positive effect against 9 isolates with low inhibition zone ranged from 11 - 16.5 mm.

The highest activity was recorded with *C. compressa* extract against *S. aureus* 287, and *B. cereus* 4 with zones of inhibition 20.5 and 22 mm respectively ($P < 0.05$). Moderate activity was obtained against *S. aureus* 122, *B. cereus* 16 and *B. cereus* 72 with zones of inhibition 18.5, 20.5 and 17.5 respectively. Weak activity was obtained against *S. aureus* 128 with zones of inhibition of 14 mm (Table 3). In comparison, flavonoids extracted from *P. pavonica* exhibited the highest activity against B2 with zone of inhibition 16 mm and significant weak activity against *S. aureus* 122, *S. aureus* 128 and *S. aureus* 287 with zones of inhibition 13.5, 11.5 and 11.5 mm ($P < 0.05$) (Table 3).

Table 4 showed the antibacterial activity of the flavonoids extract of *C. compressa* against all tested Gram negative isolates in which zones of inhibition varied from 15 to 31mm while *P. pavonica* showed lesser or no activity with zone inhibition diameter (14.5 and 27 mm). The highest significant zones of inhibition was observed in *C. compressa* extract (31 ± 1 mm) against *S. enterica* 17 ($P < 0.05$) followed by *S. enterica* 29 (24 mm). Also it was active against *S. enterica* 18, *S. enterica* 19 and EHEC O157 57 zones of inhibition (17 mm) followed by EHEC O157 55, EHEC O157 49 (16 mm). In addition, *C. compressa* extract recorded the lowest activity against EHEC O157 52 (15 mm) (Table 4). In comparison, *P. pavonica* extract was active against *S. enterica* 17 (14, 5) and S. *enterica* 29 (27 mm) and the rest of isolates were resistant.

The MAR and ARI indices of the tested extracts were reported in Table 3 and Table 4. Unlike tested antibiotics, most isolates exhibited susceptibility to flavonoids extracted from algae with an average MAR index of 0 - 0.5 which indicates that tested algal extracts are effective (Table 5 and Table 6). As shown in Table 5 and Table 6, *S. aureus* 122, *S. aureus* 128, *S. aureus* 287, *B. cereus* 16, *S. enterica* 17, and *S. enterica* 29 isolates with very low MAR value (0.0) were found to be susceptible to both extracts, in contrast *S. aureus* 125, *S aureus* 283 and *B. pumilus* 124 isolates showed significantly high resistance with higher MAR value (1). Over all, the results indicated that: *B. cereus* S. *enterica* and EHEC O157 isolates showed greater resistance with higher ARI indices (0.5) than *S. aureus* isolates with a value ARI index of 0.41 (Table 3 and Table 4).

3.2. Minimal Inhibitory/Bactericidal Concentrations (MICs/MBCs) of Active Extracts

The minimal inhibitory concentration (MIC) of flavonoids extracted from *C. compressa* and *P. pavonica* were determined for various tested organisms. The MICs ranged from 31.25 µg/mL to 125 µg/mL and 62.5 µg/mL to 500 µg/mL, respectively. The MICs and MBCs of the extracts are presented in Figure 2 and Figure 3.

The flavonoids extract of *C. compressa* had the lowest MIC value, 31.25 µg/ml and the lowest MBC value 62.5 µg/ml against *S. aureus* 122 and *S. enterica* 17; MIC value of 62.5 µg/ml and MBC value of 125 µg/ml against *B. cereus* 4

Table 5. MBC/MIC ratio for tested bacterial strains against flavonoids algal extracts.

	C. compressa	*P. pavonica*
	MBC/MIC	
	***S. aureus* isolates**	
S1	2	2
S2	2	2
S3	4	3
	***B. cereus* isolates**	
B1	2	-
B2	3	2
B3	2	-
	S. *enterica* isolates	
Sal 1	2	2
Sal 2	4	-
Sal 3	2	-
Sal 4	4	3
	EHEC O157 *isolates*	
E1	4	-
E2	4	-
E3	4	-
E4	4	-

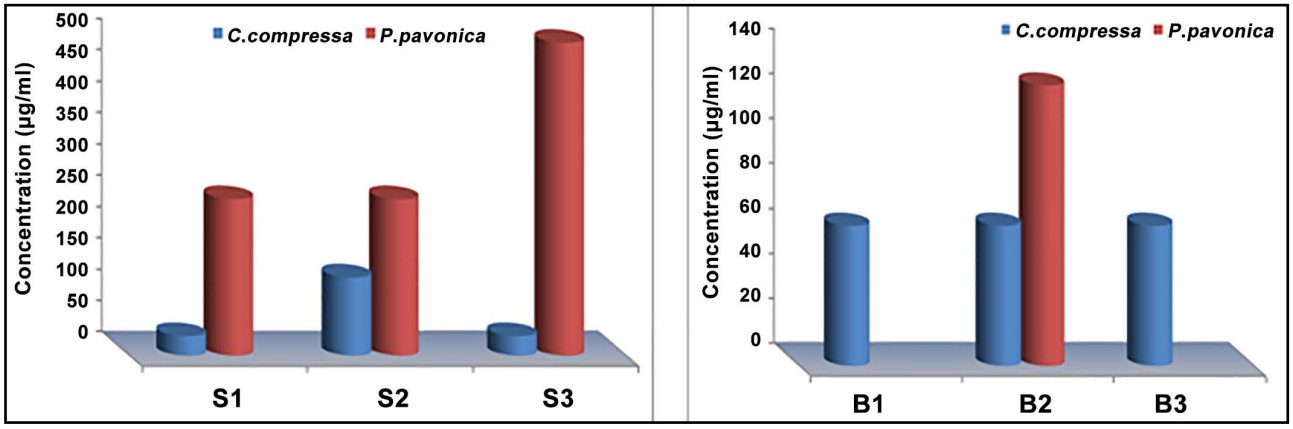
S1: *S. aureus* 122; S2: *S. aureus* 128; S3: *S. aureus* 287; S4: *S. aureus* 125; S5: *S. aureus* 283 ; B1: *B. cereus* 4; B2: *B. cereus* 16; B3: *B. cereus* 72; B4: *B. pumilus* 124; Sal 1: *S. enterica* 17; Sal 2: *S. enterica* 18; Sal 3: *S. enterica* 19; Sal 4: *S. enterica* 29; E1: EHEC O157 57; E2: EHEC O157 55; E3: EHEC O157 52; E4: EHEC O157 49.

Table 6. Flavonoid, and Proanthocaynidin content in flavonoids extracted from *C. compressa* and *P. pavonica*.

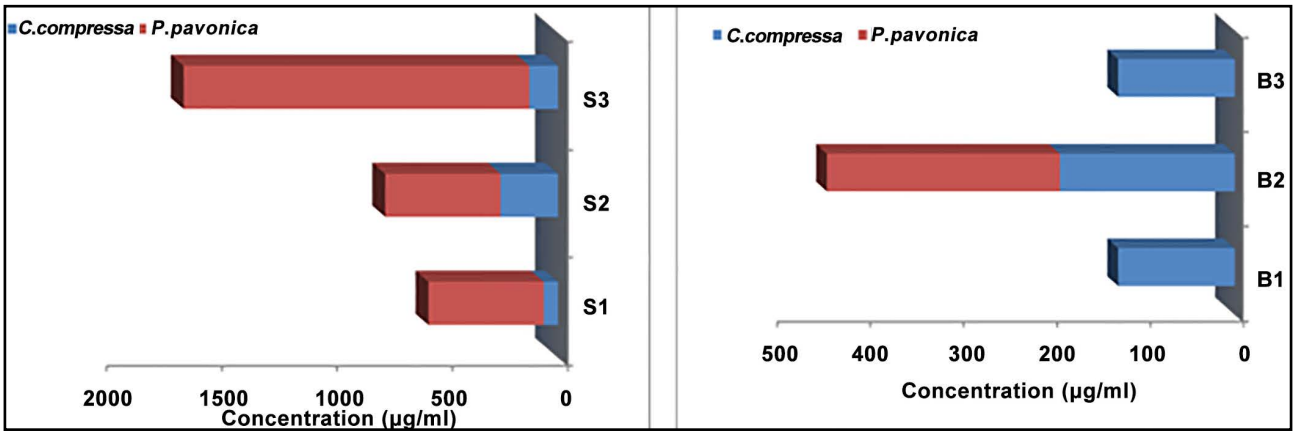
Algae	TFC*	Proanthocyanidin**
C. compressa	110.92 ± 11.38^{a}	0.24 ± 0.0^{a}
P. pavonica	70.08 ± 2.42^{b}	0.072 ± 0.0^{b}

Each value is represented as mean ± SD (n = 3). Means with the same letter are not significantly different at $P < 0.05$. *: Expressed as mg Rutin/g, ** Expressed as mg Catechin/g.

and *B. cereus* 72, MIC value of 125 µg/ml and MBC value of 250 µg/ml against *S. aureus* 128 and S. *enterica* 19, MIC value of 62.5 µg/ml and MBC value of 187.5 µg/ml against *B. cereus 16*, while MIC value of 62.5 µg/ml and MBC value of 250 µg/ml against *S. enterica* 29. On the other hand, the highest MIC value of 125 µg/ml, and the highest MBC value of 500 µg/ml due to *C. compressa* extract were noted for EHEC O157 57, EHEC O157 55 and *S. enterica* 18 (Figure 2 and Figure 3).



(a)



(b)

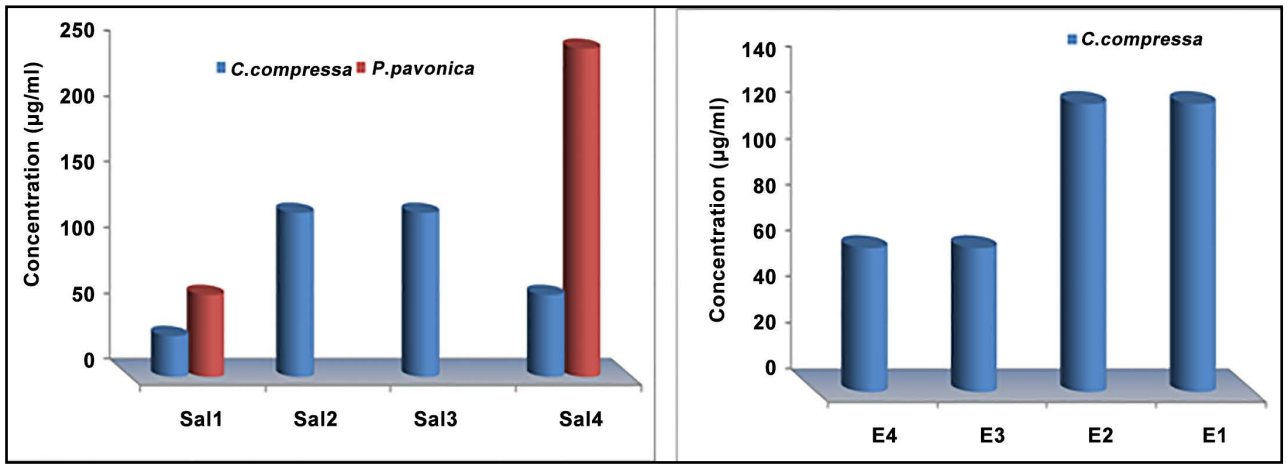
Figure 2. Minimal inhibitory concentration (MIC) (a) and minimal bactericidal (MBC) (b) of the algal extracts against Gram positive bacteria (S1: *S. aureus* 122; S2: *S. aureus* 128; S3: *S. aureus* 287, B1*: B. cereus* 4; B2: *B. cereus* 16; B3: *B. cereus* 72).

The flavonoids extract of *P. pavonica* showed the lowest MIC value of 62.5 µg/ml and the lowest MBC value of 125 µg/ml against *S. enterica* 17; MIC value of 125 µg/ml and MBC value of 250 µg/ml was recorded against *B. cereus* 16; MIC value of 250 µg/ml and MBC value of 500 µg/ml was noted against *S. aureus* 122 and *S. aureus* 128; MIC value of 250 µg/ml and MBC value of 750 µg/ml were recorded against *S. enterica* 29 while the highest MIC value of 500 µg/ml, and the highest MBC value of 1500 µg/ml was obtained against *S. aureus* 287 (Figure 2 and Figure 3).

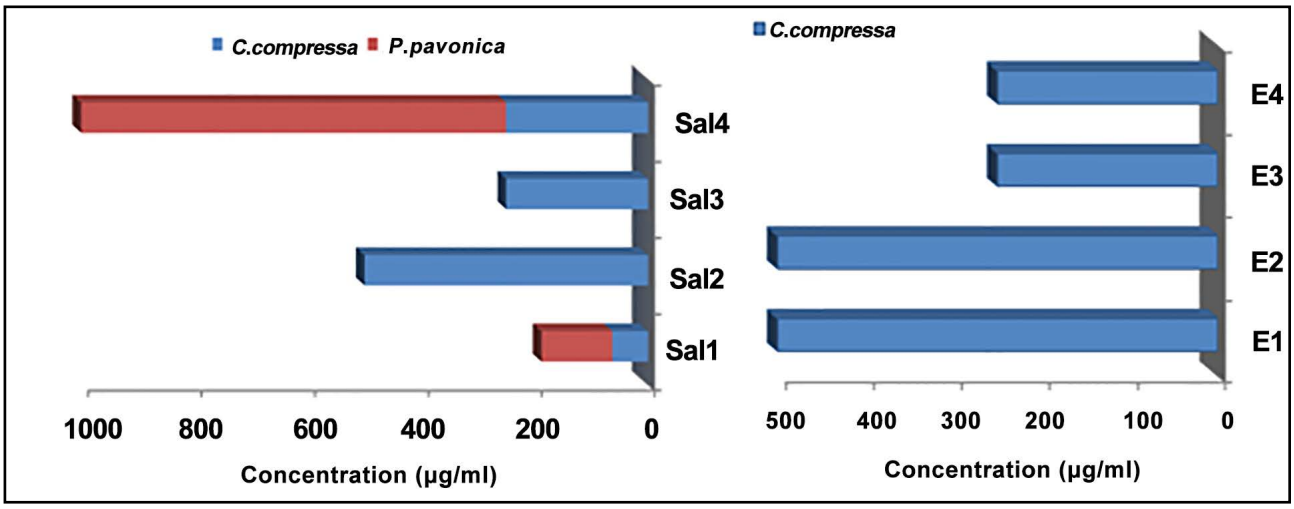
Flavonoids extracted from *C. compressa* also displayed the best spectrum of bactericidal effect with a ratio MBC/MIC ≤4 obtained on all tested bacterial strains (Table 5). Although an antibacterial activity (MIC) was detected in the flavonoids extracts of two algae against all 11 tested bacterial isolates, the MBC values (bactericidal activity) showed a different pattern of activity from that of MIC (Table 5).

3.3. Flavanoids and Proanthocyanidin Contents

Flavonoids including proanthocyanidin were determined in tested algae. Their



(a)



(b)

Figure 3. Minimal inhibitory concentration (MIC) (a) and minimal bactericidal (MBC) (b) of the algal flavonoids extracts against Gram negative bacteria (Sal 1: *S. enterica* 17; Sal 2: *S. enterica* 18; Sal 3: *S. enterica* 19; Sal 4: *S. enterica* 29; E1: EHEC O157 57; E2: EHEC O157 55; E3: EHEC O157 52; E4: EHECO157 49).

levels in *C. compressa* extracts were remarkably higher than their counterpart in *P. pavonica* ($P < 0.05$). The amount of flavonoids in *C. compressa* was 110.92 mg/g Rutin equivalent while in *P. pavonica* was 70.08 mg/g Rutin equivalent (Table 6).

3.4. Correlation between the Values of Antibacterial Activities of Flavonoids and Proanthocyanidin Contents

In order to examine possible association between antibacterial activity and flavonoids and proanthocyanidin content in algae extracts, correlation coefficient (R^2) was evaluated.

There were no correlations between the level of flavonoids with antibacterial activity against *B. cereus* isolates and *S. enterica* isolates whereas no correlation was observed between proanthocyanidin content and antibacterial activity against EHEC O157 isolates. In addition, a positive correlation was found between each

of flavonoids and proanthocyanidin contents in algae extracts with antibacterial activity against *S. aureus* isolates and EHECO157 isolates (R^2 = 0.646, 0.99 respectively).

4. Discussion

Antimicrobial resistance is a growing problem and a public health threat. To overcome infections caused by multidrug-resistant strains, new classes of antimicrobials should be developed. Algae may offer an alternative source of antimicrobial agents with significant activities against pathogens with less risks of adverse effect encountered with synthetic antibiotics. Moreover, a number of antibiotics have lost their effectiveness due to the abuse of their applications and the evolution of resistant strains of microorganisms [40].

Marine organisms including macroalgae are a rich source of structurally novel biologically active metabolites [2]. Therefore, active constituents of various algae extracts could be potential bioactive compounds of interest in the pharmaceutical industry [1]. In the present study, phytochemical screening of the seaweeds showed the presence of most important phytoconstituents. Flavonoids were present in *C. compressa* in higher amounts than those found in *P. pavonica*. Since the preliminary screenings of the algal extract have shown the presence of flavonoids in appreciable amounts [41], this fraction was chosen to be evaluated for antibacterial activity.

Flavonoids belong to the large group of secondary metabolites called polyphenols with variable phenolic structures and are found to be the most important natural substances responsible for their bioactivity. Flavonoids have been reported to exhibit a wide range of bioactivity including antioxidants, antibacterial, antiviral, anticancer, anti-inflammatory, anti-allergic and also as potential therapeutic agents against a wide variety of diseases [6] [7] [8].

It has been stated that antibacterial activity depends on algal species, the efficiency of the extraction method, and the resistance of the tested bacteria [42]. Many studies revealed that brown algae has higher amount of flavonoids compared with green and red algae [43] [44]. Hence, in this study, brown algae, namely *C. compressa and P. pavonica,* were selected to evaluate their flavonoids contents and study their antibacterial activity against isolated multidrug resistant (MDR) bacteria.

The extraction method plays an important role in the overall effect of natural antimicrobial products. Active compounds have been extracted and purified using different extraction methods including ultrasonication assisted extraction and soxhlet extraction [16] [17]. However, these methods require enormous amounts of solvents, time consuming, expensive and environmentally unfriendly. Therefore, microwave was used to assisted flavonoids extraction which was approved to have high efficiency for extraction [45]. The flavonoids yields obtained with microwave assisted extraction were far highly active than conventionally extracted ones [17]. Higher flavonoids yields per gram of algae material were obtained in microwave assisted extraction in both tested samples. The yield ob-

tained from *C. compressa* was higher than that in *P. pavonica*, which was consistent with our previous findings [46].

Antibiotics provide the main basis for the therapy of bacterial infections. However, the high genetic variability of bacteria enables them to rapidly evade the action of antibiotics by developing antibiotic resistance. As resistance becomes more common, there becomes a greater need for alternative treatments. However, despite a push for new antibiotic therapies there has been a continued decline in the number of newly approved drugs [47] [48]. Our results showed that the isolates were resistant to at least 7 different antibiotics, which indicates that all isolates are multi-drug resistant [49]. Gram negative isolates were highly resistant to antibiotics and showed significantly greater resistance with higher MAR indices (0.63 - 0.96) and ARI index (0.18 - 0.19). In comparison Gram positive isolates including MRSA were more susceptible with lower MAR index ranged from 0.38 - 0.83 and ARI index (0.11 - 0.15). The isolates that have MAR index ≥0.2 were considered as multidrug resistant [39]. Therefore, we aimed to analyze the flavonoids extracted from seaweeds for their use as new antibacterial agents against multidrug resistant bacteria which have been isolated from food products.

The antibacterial activity of crude extracts of *C. compressa and P. pavonica* are well known [21] [24] [50] [51]. However, no study has determined the antimicrobial activity of flavonoids extracted from brown algae so far. In the present study, flavonoid rich extract of *C. compressa* had the broadest inhibitor activities against the test isolates (highest inhibitor activity against *S. enterica* 17, 31 mm DIZ, MIC, 31.25 µg /ml, MBC 62.5 µg /ml). In comparison flavonoids extracted from *P. pavonica* had weak effect on most tested isolates (most active against *S. enterica* 17, 27.5 mm DIZ, MIC, 62.5 µg /ml, MBC, 125 µg/ml). Among the tested bacterial strains, EHEC O157 isolates (a food borne pathogen) was found most sensitive against the *C. compressa* extracts with DIZ ranged from 15 to 17 mm. while *P. pavonica* showed no effect on all EHEC O157 isolates, which was in agreement with [16].

The minimum concentration necessary to kill an organism should be equal to or greater than the MIC for that microbe [52]. A sample is bactericidal when the ratio MBC/MIC ≤ 4 and bacteriostatic when this ratio is >4 [53]. It therefore seems to be that antibacterial effects obtained with the flavonoids extracted from tested algae against susceptible isolates, proved to have bactericidal activity. Current results were in parallel with previous results which suggested that flavonoids are capable of bactericidal activity which resulting mostly from the impairment of the cell wall integrity and to cell agglutination [54].

The different rates of inhibition activities appear to be directly related to the qualitative and quantitative amount of the extracted flavonoids content in tested algae. The amount of flavonoids in *C. compressa* was 110.92 mg/g Rutin equivalent which was higher than that reported by [55]. The recorded antibacterial activity was shown to correlate with the total flavonoids and proanthocyanidin contents in the tested algal extracts with high correlation coefficient (r) ranged

from 0.293 to 0.995. The antimicrobial properties of natural products have been attributed to its high flavonoids content and in particular the presence of the proanthocyanidin [8]. The present results showed that flavonoids rich extract of *C. compressa* exhibited a significant inhibitory activity against both Gram positive and negative bacteria which increased its value as an ideal or broad spectrum antibacterial for MDR microorganisms.

Our results are in agreement with several previous findings demonstrating greater activity of flavonoids extracts towards Gram-positive bacteria compared to Gram-negative bacteria [56]. The probable reason is the difference in the composition and permeability of their cell walls. The cell walls of Gram-positive bacteria are made of peptidoglycans and teichoic acids, while the outer membrane found in the Gram-negative cell wall is composed of structural lipopolysaccharides which render the cell wall impermeable to lipophilic solutes [57] [58].

To the best of our knowledge, there are no reports concerning the comparative antibacterial activity analysis for investigated flavonoids extracted from algae. However, many studies have been done on flavonoids extracted from plants [59] [60] [61]. It has been revealed that the antibacterial activities of natural products are related to structures and cellular membranes of tested bacteria by varied mechanisms via several events at a cellular level [62] [63]. Previous study showed a strong relationship between flavonoids structures due to the substitutions on the aromatic rings and antibacterial activity [64].

Flavonoids have proven to be antibacterial agents, especially the ones with hydrophobic substituent such as prenyl groups, alkylamino chains, alkyl chains, and nitrogen or oxygen containing heterocyclic moieties [65] [66]. Flavones have been widely investigated with respect to their antibacterial activity showed strong activities against both Gram negative bacteria (*E. coli*, *S. typhimurium*) and Gram positive bacteria (*S. epidermis*, *S. aureus*). Another observation showed limited activities against Gram positive bacteria [67]. Proanthocyanidins also have been reported to be effective antibacterial agents against both sensitive and multi-drug resistant strains [68] [69].

The exact mode of action mechanism of natural antimicrobials is still not fully understood. However, different mechanisms for different antimicrobial groups have been reviewed [8], and concluded that , different versions of proposed mechanisms of action for antibacterial activity of flavonoids into three main mechanisms including inhibition of nucleic acid synthesis [70], inhibition of cytoplasmic membrane function [54], inhibition of energy metabolism by disturbing the exchange of nutrients and metabolites [71] in addition, inhibition of cell membrane synthesis and the aggregatory effect on whole bacterial cells was considered as possible mechanism [72].

5. Conclusion

This study concluded that the flavonoids isolated from Libyan algae showed antibacterial potentiality against multi-drug resistant Gram positive and negative

bacterial isolates including MRSA. However, whether such extracts will act as effective antibacterial agents *in vivo* remain to be investigated and the study of mechanisms of actions is necessary prior to their application.

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Competing Interests

Authors have declared that no competing interests exist.

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