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Research Article

Antibacterial Activity of Crude Extracts and Phlorotannin Isolated from the Diatom *Cymbella* spp.

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ABSTRACT

Antibacterial activity of organic and aqueous extracts from the diatoms *Cymbella* spp. and it's isolated phlorotannins, collected from Iraq were carried out against seven different species of pathogenic microorganisms: *Staphylococcus aureus*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumonia*, *Salmonella typhi* and *Pseudomonas aeruginosa* using disc diffusion and microdilution assay. All extracts showed antibacterial activity against most tested bacteria except, *P. mirabilis*. The highest growth inhibition was seen against *P. aeruginosa* (zone of inhibition: 23 mm in diameter) using the ethanol extract concentration 50 mg/ml; and the highest MIC value was observed against *P. aeruginosa* (MIC=0.19 mg/ml) using the same extract. The isolated compound (phlorotannin) with a concentration of 50 mg/ml achieved good antibacterial activity against all tested bacteria (zone of inhibition range: 9-25 mm) and MIC values in range: 1.56 - >3.12 mg/ml.

Key words: Diatoms, Antibacterial activity, Phlorotannin

INTRODUCTION

Freshwater micro algae comprise a vast group of photosynthetic, heterotrophic organisms, which have an extraordinary potential for cultivation as energy crops. They are able to produce a wide range of commercially interesting by products such as fats, oils, sugars and functionally bioactive compounds.

The algae belong to a group of organisms that has enormous ecological importance and represents a significant proportion of the world's biodiversity. Apart from being a source of food, seaweeds are also used as fertilizers, and nitrogen-fixing cyanobacteria have been used to increase rice yields. The main class of commercially valuable algae products are the algal polysaccharides, but algae also produce a range of unique secondary metabolites, many of which have specific biological activities(1,2,3,4).

Discovering new therapeutic molecules is becoming increasingly important as more and more bacteria become resistant to the commonly used antibiotics. Traditionally used in Asiatic medicines, algae, since the second half of the 20th century, have been screened for their biological activities. Thus, antibacterial effects have been noticed in all the algal classes and notably in diatoms, the major component of the phytoplankton(5,6,7,8).

However, most of these antibiotic activities have only been tested against human pathogens and the active molecule(s) have rarely been purified. The antibacterial activities of marine algae have been well documented(9,10).

Secondary metabolites from marine algae have been reported as interesting sources of pharmacological compounds [(11)] and have an important role in marine ecology such as plant herbivore interactions, toxicity for defense, space competition and antifouling(12,13).

Marine organisms are a rich source of structurally novel and biologically active metabolites(14,15) . Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry (16). To date, many chemically unique compounds of marine origin with various biological activities have been isolated, and some of them are under investigation and are being used to develop new pharmaceuticals (16,17). The cell extracts and active constituents of various algae have been shown to have antibacterial activity in vitro against Gram-positive and Gram-negative bacteria(12).

Biogenic compounds belonging to several classes of marine micro and macro algae have been identified over the last few decades, and their chemical constitution and pharmacological activity have been studied in detail(18,19,20,21).

In the marine ecosystem, phlorotannins, common secondary metabolites from marine brown algae based on units of phloroglucinol, affect marine herbivores as feeding deterrents, influencing assimilation efficiency and defending against fouling organisms(22). Phlorotannins, which are oligomers of phloroglucinol, have been reported to be both anti-plasmin inhibitors(23,24) and antioxidants(25). They have also been found to have algicidal activities against red tide micro algae.

The present study was undertaken in order to examine the antibacterial effects of *Cymbella* spp. extracts and isolated crude phlorotannins against some pathogenic bacteria.

MATERIALS AND METHODS

Collection and isolation:

Cymbella spp. samples were collected from Al

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Shallalat River on July 2007, Nineveh province, Iraq and identified by Dr. Youssif Al-Shahiri, Department of Biology, College of Education, University of Mosul. Epiphytes and necrotic parts were removed from the samples after rinsing and cleaning with sterile water.

***Preparation of extracts*:**

Powdered samples (100g) were extracted with water, ethanol, chloroform and finally acetone using a soxhlet extractor. Each extraction was carried out for 8-10 h continuously. The solvents were then removed using a rotary vacuum evaporator at 40 °C to give concentrated extracts, which were frozen and freeze-dried until use.

***Preparation of crude phlorotannin*:**

Powdered samples (80g moisture ~10%) were extracted using methanol (240 ml) with shaking (90 rpm) at 5°C for 48 h. The extracts were concentrated in vacuo and transferred to separatory funnel. Methanol (240 ml), chloroform (480 ml) and deionized water (180 ml) were added. Two layers were separated, upper and lower(26).The upper layer was extracted twice with ethyl acetate (300 ml) and the resulted fraction was evaporated in vacuo. The extract here is referred to as crude phlorotannins which, was identified using Thin-layer chromatography (TLC)(27).

***Preparation of extract concentrations*:**

A volume of (0.25g) from each extract (ethanol, water, chloroform and acetone) was dissolved in 5ml dimethylsulfoxide "DMSO" to produce a final concentration of 50 mg/ml which was used as a stock concentration in providing next dilutions (25, 12.5, 6.25 and 3.12 mg/ml), all extracts were sterilized by filtration through a 0.45 ìm membrane filter(28).

***Test bacteria*:**

The following microorganisms were used as test organisms: *Staphylococcus aureus*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumonia*, *Salmonella typhi* and *Pseudomonas aeruginosa*. All microorganisms were clinical isolates obtained from the bacterial collection of Department of Biology, College of Science, University of Mosul, Iraq. Microorganisms were further identified by current standard microbiological methods according to, (29) to insure.

***Screening of antibacterial activity*:**

The diatom *Cymbella* spp. extracts and phlorotannins were screened for their antibacterial activity using the disc diffusion assay described by (30).100 ìl of prepared culture was spread on surfaces of Mueller–Hinton agar (MHA). Sterile filter paper discs (Whatmann No.1: 6mm in diameter) were soaked with different concentrations of both extracts and compound (0.1 ml of extract/10 paper disc), then placed on the surface of the inoculated media plates slightly, antibiotic discs (Bioanalyse) 6 mm in diameter of (Gentamycin 10µg and Cefalexin 30µg)were used as positive controls. Spread plates were then kept at room temperature for 30 min to allow diffusion and absorption of extracts prior to incubation at 37°C for 24 hours. At the end of the period, inhibition zones formed on the medium were evaluated in mm.

***Microdilution assay*:**

The minimal inhibitory concentration (MIC) of the diatom extracts and phlorotannins were determined based on a microdilution method in 96 multi-well microtiter plates, as previously described(31)Briefly, the dissolved materials were first diluted to the highest concentration to be tested (3.12 mg/ml), 50 ìl of Nutrient broth was distributed from the 2nd to the 9th well, a volume of 100 ìl from each of the extract and compound initially prepared was pipetted into the 1st test wells of each microtiter line, and then 50 ìl of scalar dilution was transferred from the 2nd to the 9h well. To each well was added 10 ìl of resazurin indicator solution (prepared by dissolving a 270-mg tablet in 40 ml of sterile distilled water). Finally, 10 ìl of bacterial suspension was added to each well. The final concentration of the extracts adopted to evaluate antibacterial activity was included from 3.12 mg/ml to 0.006 mg/ml. Two columns in each plate were used as positive controls containing antibiotics (gentamycin and cefalexin in serial dilutions of 3.12-0.006 mg/ml). Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37 °C for 18-24 h. Color change was then assessed visually. Any color change from purple to pink or colorless was recorded as positive.The lowest concentration at which color change occurred was taken as the MIC value. The average of 3 values was calculated and that was the MIC for the test material.

RESULTS

Results of antibacterial screening from *Cymbella* spp. extracts are presented in Table 1. Inhibitory effects were between very high effects and no activity. *P. aeruginosa* showed highest susceptibility against the aqueous extract using the extract concentration 50 mg/ml and a direct relation was seen between inhibitory effects and extract concentrations. Meanwhile, the weakest aqueous extract action was seen against *E. coli* with an inhibition zone of 11 mm. Ethanol extract revealed promising results against most tested bacteria. Very clear inhibition zones were observed against *P. aeruginosa*, *C. diphtheriae* and *S. aureus* (23, 22 and 20 mm respectively) followed by *K. pneumonia*, *S. typhi* and finally *E. coli*.

The acetone extract had moderate antibacterial activity against all tested bacteria except *E. coli* and *P. mirabilis*. Inhibition zones observed were between 7-17 mm in diameter and the highest effect was against *P. aeruginosa* using the extract concentration 50 mg/ml.

The chloroform extract demonstrated good inhibitory results against most tested bacteria and the highest growth inhibition (16 mm) was seen against *C. diphtheriae* using the highest extract concentration. Furthermore, *P. mirabilis* re-

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Table 1: Antibacterial activity of different extracts from diatoms

Microorganisms	Extract type	Zone of inhibition (mm) Extract concentrations (mg/ml)					Control (µg/ml)	
		50	25	12.5	6.25	3.12	G	C
S. aureus	Aq	16	14	11	9	8	20	N.T
	E	20	16	13	11	9		
	A	13	12	10	9	7		
	C	11	9	8	–	–		
C. diphtheriae	Aq	19	17	15	13	9	20	28
	E	22	21	19	17	15		
	A	15	13	10	9	–		
	C	16	14	12	10	8		
E. coli	Aq	11	10	9	7	–	N.T	33
	E	13	11	10	9	8		
	A	–	–	–	–	–		
	C	1	9	8	7	–		
P. mirabilis	Aq	–	–	–	–	–	N.T	27
	E	–	–	–	–	–		
	A	–	–	–	–	–		
	C					–		
K. pneumonia	Aq	13	12	10	8	7	N.T	32
	E	16	14	12	11	9		
	A	15	13	12	10	9		
	C	12	11	10	8	–		
S. typhi	Aq	18	17	15	14	13	27	N.T
	E	14	13	11	10	9		
	A	16	14	13	11	10		
	C	10	9	8	7	–		
P. aeruginosa	Aq	22	21	20	18	17	31	N.T
	E	23	22	20	18	17		
	A	17	16	15	13	11		
	C	15	14	12	11	10		

Aq: Aqueous, E: Ethanol, A: Acetone, C: Chloroform, G: Gentamycin (10 µg), C: Cefalexin (30 µg), N.T: Not tested, –: No activity.

Table 2: Antibacterial activity of phlorotannin isolated from diatoms

Microorganisms	Zone of inhibition (mm) Phlorotannin 50 mg/ml
S. aureus	23
C. diphtheriae	25
E. coli	15
P. mirabilis	9
K. pneumonia	19
S. typhi	20
P. aeruginosa	25

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Table 2: Minimal inhibitory concentrations of diatom extracts and phlorotannin

Microorganisms	MIC values (mg/ml)						
	Extracts				Phlorotannin	Control	
	Aq	E	A	C		G	C
S. aureus	0.78	0.78	0.78	>3.12	1.56	0.02	N.T
C. diphtheriae	0.78	0.39	>3.12	0.78	1.56	0.02	0.01
E. coli	>3.12	0.78	>3.12	>3.12	3.12	N.T	0.01
P. mirabilis	>3.12	>3.12	>3.12	>3.12	>3.12	N.T	0.01
K. pneumonia	0.78	0.78	0.78	>3.12	3.12	N.T	0.01
S. typhi	0.39	0.78	0.78	>3.12	1.56	0.01	N.T
P. aeruginosa	0.19	0.19	0.78	0.78	1.56	0.01	N.T

Aq: Aqueous, E: Ethanol, A: Acetone, C: Chloroform, G: Gentamycin, C: Cefalexin

sisted all aqueous and organic extract concentrations.

The crude isolated compound (phlorotannin) with a concentration of 50 mg/ml achieved good antibacterial activity against all tested bacteria (Table 2). The best zone of inhibition was seen against *C. diphtheriae* and *P. aeruginosa* (25 mm in diameter). *P. mirabilis* showed least susceptibility against the compound and was inhibited with a zone of inhibition (9 mm in diameter).

Table 3 summarizes the MIC values of the different extracts and the compound phlorotannin isolated from *Cymbella* spp.. Among the tested extracts, the aqueous and ethanol extracts showed best MIC values against most tested bacteria. The strongest activity was against *P. aeruginosa* with a MIC value of 0.19 mg/ml. In contrast, chloroform extract was only active against *C. diphtheriae* and *P. aeruginosa* with a MIC value of 0.78 mg/ml. The best MIC value (0.78 mg/ml) achieved by the acetone extract was against *S. aureus, K. pneumonia, S. typhi* and *P. aeruginosa*. Moreover, phlorotannin revealed good MIC values against most tested bacteria and ranged between 3.12-1.56 mg/ml.

The standard drugs gentamycin and cefalexin were active against all reference bacteria (zone of inhibition range: 20-31 mm; MIC range: 0.02-0.01 mg/ml).

DISCUSSION

The activity of plant extracts against bacteria have been studied for years, but in a more intensified way during the last 3 decades. During this period, numerous antimicrobial screening evaluations have been published based on the traditional use of Chinese, African, and Asian plant-based drugs(32).In the present study organic and aqueous extracts from diatoms inhibited all bacterial growth except *P. mirabilis*. The zones of inhibition ranged from 7-23 mm using the disc diffusion method. It is possible that the extracts, which did not show any inhibitory activity at the tested concentrations, might show inhibition of bacterial growth if used in higher concentrations as the actual active compound (s) present in these extracts may be of very minute quantities. Furthermore, the ethanol extract had promising MIC values against most tested bacteria and the best MIC value was seen against *C. diphtheriae*.

The isolated compound phlorotannin had good antibacterial activity against all tested bacteria. The mode of action may be related to its ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins, etc. They also complex with polysaccharide(33). The Gram-negative bacteria *P. mirabilis* resisted all organic and aqueous extracts and was only inhibited using 50 mg/ml of phlorotannin. The resistance of bacteria towards different drugs can be due to modification of the target site, bypass of pathways, decreased uptake (reduced intracellular concentration of the antimicrobial agent, either reducing membrane permeability or by active efflux pump), enzymatic inactivation or modification of the drug, or overproduction of the target(34).

It can be concluded that different extracts and phlorotannin isolated from diatoms had excellent antibacterial activities against seven different bacterial species and that phlorotannin is very much responsible for the antibacterial activity of the diatom extracts. Additional in vivo studies and clinical trials would be needed to justify and further evaluate the potential of this compound as an antibacterial agent in topical or oral applications.

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