Diketopiperazine derivatives from *Enterobacter cloacae* isolated from the Red Sea alga *Cystoseira myrica*

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Aim

This study is an attempt to explore the biological activities of isolated endophytic bacteria from marine sources that were coded A1, A2, and A3 (*Padina pavonica*), A4 (*Cystoseira myrica*), A5 (*Acanthophora dendroides*), and A6 (*Sargassum sabrepandum*). The bacteria coded C1, C2, and C3 were isolated from the soft coral Nephthea mollis and S1 and S2 were isolates from the sponge *Hymedesmia* spp. The primary aim of the study was the identification of active compounds.

Materials and methods

The bioactive compounds were extracted using ethyl acetate from nutrient broth media; biological activities of the extracted metabolites and 16S rDNA identification of the most promising isolate were studied. The eight major fractions of the extract showed different composition patterns when identified by liquid chromatography/mass spectrometry analyses.

Results and conclusion

Agar diffusion assay showed inhibitory activities of A4 extracts against the growth of most pathogenic microorganisms. Identification using PCR 16S rDNA and electrophoresis confirmed 98% identity to the *Enterobacter cloacae* strain GH1 (ac: JF261136.1). Eight compounds out of fifteen in the extract were identified as diketopiperazine derivatives. The maximum growth of *E. cloacae* was obtained at 30°C, pH 7, with the addition of maltose and KI to the media. The free radical scavenging activity exhibited good antioxidant activity (72.19%, IC_{50} = 1.266 mg/ml) on using 2.0 mg/ml of the crude extract. The extract showed a high antiviral activity towards Newcastle disease virus and avian influenza virus A(H5N1).

Keywords:
antimicrobial activity, antioxidant, antiviral, diketopiperazines, *Enterobacter cloacae*

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Introduction

Marine organisms are an inexhaustible source of useful chemical substances for the development of new drugs; among these organisms are marine algae, which are capable of biosynthesizing a broad range of secondary metabolites, and bacteria that live in oceans. *Enterobacter cloacae* is a rod-shaped, Gram-negative bacterium that frequently grows at 30°C on nutrient agar medium (NAM) or nutrient broth medium [1]. It is a member of the normal gut flora in many humans [2] and has been used as a biological control in plant diseases such as those caused by the seed-rotting oomycete *Pythium ultimum*. It is also used to control insect pests on mulberry leaves and suppress disease [3].

Many of the marine algae species are often accompanied by several bacterial strains that are obtained from the sea together with the algae cells, or are the result of contamination of the algae culture. These mixed populations that are present in the culture and in the sea show that the bacteria use organic substances secreted by living or dead algae cells. It has been observed that many types of seaweed thrive in the presence of bacteria than in their absence. Some seaweed species need vitamins for their growth and possibly the bacteria are partially responsible for the production of these substances; some of them even produce antibiotics [4,5]. Because of the highly chemical and harsh physical conditions in the marine environment, the organisms produce a variety of molecules with unique structural features and exhibit various biological activities. The majority of the marine natural products have been isolated from sponges, coelenterates (sea whips, sea fans, and soft corals) [6].

As antibiotic resistance has developed, medical researchers have fought back with alternative antibiotics and combination therapy, but constant overuse of antibiotics in humans and livestock have lead to bacterial strains becoming resistant to nearly all known antibiotics. Hence, constant effort is required to discover newer antibacterial agents that are effective against pathogenic bacteria that are resistant to currently available antibiotics [7].

Materials and methods

Collection and identification of samples

Sponge, soft coral, and seaweeds were collected from the Red Sea at a depth of 5–8 m from the coast of Rass Mohammed (South Sinai, Egypt) and at a depth of 2 m from the Institute of Marine Science (Hurghada, Egypt) in March 2010. The samples were washed with tap water and identified by the Coral Reef Ecology and Biology Group, National Institute of Oceanography and Fisheries (Suez, Egypt).

Isolation and maintenance of endophytic isolates

The marine organism was soaked in distilled water, drained, and then rinsed in 70% ethanol for 30 s, followed by wash in sterile water; tissues were cut into 0.5–1 cm pieces. The sterile pieces were placed on the surface of NAM containing the following components (g/l): glucose 10, peptone 6, yeast extract 3, meat extract 1.5, and agar 25. The media were incubated for 28 h at 30°C. On the basis of morphological features, colonies were picked, purified, and maintained on the NAM.

Extraction of bioactive compounds

The isolated bacteria were inoculated in 20 l nutrient broth medium at 30°C under shaking conditions and centrifuged after 4 days. The filtrate and the separated cell mass were extracted three times by ethyl acetate and evaporated until they were completely dry using a rotavapor with heated waterbath up to 40°C. The crude extract was weighed and tested for biological activities.

Pathogenic organisms

All the tested organisms were supplied by the Biotechnological Research Center, Al-Azhar University (Cairo, Egypt); the bacteria were maintained on NAM and the yeast on potato dextrose agar (PDA) medium (peeled potato 200 g, dextrose 20 g, agar 18 g in 1000 ml distilled water).

Gram-positive bacteria comprised *Bacillus subtilis* (NCTC 1040), *Staphylococcus aureus* (NCTC 7447), and *Sarcina maxima* (ATCC 33910); *Gram-negative* bacteria comprised *Escherichia coli* (NCTC 10416) and *Pseudomonas aeruginosa* (ATCC 10145); and the yeast was *Candida albicans* (IMRU 3669).

In-vitro antagonism assay

A definite concentration of the crude extract (1 mg/ml) was prepared, 10 µl was used to saturate a 6 mm filter paper (Whatman 4) and was placed in Petri dishes coated with NAM for bacteria and PDA for yeast. Both media were previously inoculated with a 0.1 ml spore suspension of the pathogens [8].

The previously mentioned bacteria were incubated overnight at 37°C for 24 h, whereas yeast was cultured at 28°C for 4 days. The inhibition zones were measured in mm.

To assess the role of the salinity and components in the media on the production of bioactive metabolites, the promising isolated bacterium was cultivated in 250 ml flasks containing 100 ml of the following media:
Media I (50% seawater), containing (g/l): yeast extract 3, glucose 10, meat extract 1.5, and peptone 6.
Media II (50% seawater), containing (g/l): yeast extract 4, glucose 4, and malt extract 10.
Media III (100% seawater), containing (g/l): yeast extract 4, glucose 4, and malt extract 10.
Media IV (50% seawater), containing (g/l): peptone 5 and yeast extract 1.
Media V (100% seawater) containing (g/l): peptone 5 and yeast extract 1.

PCR 16S rDNA and electrophoresis

DNA extraction and PCR amplification of the 16S rDNA region were carried out. DNA was isolated from the isolates (A4) according to the method prescribed by Sambrook *et al*. [9]. The 16S rDNA was amplified by PCR using primers designed to amplify the 1500 bp fragment of the 16S rDNA region. The forward primer was 5′-AGAGTTTGATCMTGGCTCAG-3′ and the reverse primer was 5′-TACGGYTACCTTGTTACGACTT-3′. The PCR mixture consisted of 30 pmol of each primer, 10 ng of chromosomal DNA, 200 µmol/l dNTPs, and 2.5 U of Taq polymerase in 50 µl of polymerase buffer. The PCR was carried out for 30 cycles at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 2 min. After completion, a fraction of the PCR mixture was examined using agarose gel electrophoresis [10]. DNA sequences were obtained using a 3130X DNA Sequencer (Genetic Analyzer; Applied Biosystems, Hitachi, Japan) and BigDye Terminator Cycle Sequencing (see details below). The PCR product was sequenced using the same PCR primers. Blast program was used to assess the DNA similarities and multiple sequence alignment and molecular phylogeny were performed using BioEdit software [11].

DNA sequencing

Automated DNA sequencing based on the enzymatic chain terminator technique, developed by Sanger *et al*. [12], was performed using the 3130X DNA Sequencer (Genetic Analyzer; Applied Biosystems). The sequencing reaction was performed with four

different fluorescent labels identifying the ddNTPs instead of the radioactive labels. These fluorophores were excited with two argon lasers at 488 and 514 nm when the respective bands passed the lasers during the electrophoresis. The specific emissions were detected and the data were collected for analysis [13,14]. The thermal cycling mixture was as follows: 8 µl of BigDye Applied Biosystems, Foster City, California 94404, USA terminator mix, 6 µl of the sequencing primer (10 pmol), and 6 µl of the sample (PCR product or plasmid); the reaction was then run in the thermal cycler. The cyclic reaction consisted of 1 min at 95˚C, followed by 49 cycles of 30 s at 95˚C, 10 s at 52˚C, and 4 min at 60˚C. The products were purified using a special column according to the instructions of the manufacturer. The elute was taken and high dye formamide was added at 1 : 1/ volume ratio; the reaction was run at 95˚C for 5 min for denaturation, shock on ice, after which the sample was ready for sequencing and analysis in the 3130X DNA sequencer.

Antioxidant activity (DPPH assay)

The free radical scavenging activity using 1,1-diphenyl-2-picryl-hydrazil (DPPH) reagent was determined according to Brand-Williams *et al*. [15]. The bacterial extract was dissolved in methanol to prepare different concentrations. To each 1.5 ml of the extract sample 1.5 ml of freshly prepared methanolic DPPH solution (20 µg/ml) was added and stirred. The decolorizing process was recorded after 5 min of reaction at 517 nm and compared with a blank control.

Antioxidant activity = control absorbance – sample absorbance/control absorbance × 100%

The virus and media

Highly pathogenic avian influenza HPAI H5N1 virus A (Chicken/Egypt/9402 NAMRU 3-CLEVB 213/2007) (H5N1) of accession number Eu 623467 obtained from control Laboratory for Evaluation of Veterinary Biologics (CLEVB) was used at a titer of 106 EID_{50}/ml.

Growth medium (minimum essential medium; Gibco China (Mainland)) was supplemented with 10% new-born calf serum at pH 7.2 for primary and secondary cultures. Trypsin (1–250; Gibco) was used at a concentration of 0.23% as a cell dispersing agent. Cells of African green monkey kidney cells (Vero cells) [16] and chicken embryonic fibroblasts were cultured.

Specific pathogen-free (SPF) 9-day-old embryonated chicken eggs (ECEs) were inoculated via the allantoic sac route.

Toxicity using embryonated chicken eggs

ECEs of 9 days old (130 SPF) and six-fold dilutions of the total extract were used, 0.2 ml/ECE of each dilution was inoculated into five ECEs through allantoic sac allantoic sac and incubated at 37˚C with 80% humidity for 5 days; through all eggs candled every day.

Cytotoxicity assays

Various concentrations of the extract were added to confluent chicken embryonic fibroblast cells and Vero cell monolayer cultures 24 h after seeding and the cultures were maintained for 4 days. Then viable cell counts of each culture were determined daily by inverted microscopic inspection of cells not affected by compounds.

Hemagglutinating activity assay

Hemagglutinating activity assay was performed on the allantoic fluids of the inoculated eggs and the activity was measured by means of a microtechnique [17].

Antiviral activity

SPF ECEs were inoculated with equal volumes of Newcastle disease virus (NDV) and a nontoxic concentration after incubation of the mixture for 1 h at room temperature. The mixture was inoculated into allantoic sacs of five ECEs at 0.2 ml/ECE. Five ECEs were inoculated with the NDV, which was mixed with an equal volume of saline at 0.2 ml/ECE (positive control); another five ECEs were inoculated with saline alone at 0.2 ml/ECE (negative control). Thereafter, all the ECEs were inoculated at 37˚C and candled every 2 h until all positive control ECEs had died.

Chromatographic fractionation

Using a medium pressure liquid chromatography column, with normal silica gel 60, gradient elution was carried out starting with 100% *n*-hexane and ending with 100% ethyl acetate. Thin layer chromatography monitoring of different fractions was carried out using hexane : ethyl acetate (S1 8 : 2, S2 7 : 3, S3 5 : 5), and spots were visualized using ultraviolet and *P*-anisaldehyde/H_2SO_4 spray reagent.

Liquid chromatography/mass spectrometry analyses

High-resolution mass spectrometric (MS) data were obtained using a Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery: Thermo Scientific, Oxford, UK. coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler, and Accela pump) TUV Rheinland of

North America Inc.) under the following conditions: capillary voltage 45 V, capillary temperature 260°C, auxiliary gas flow rate 10–20 AU, sheath gas flow rate 40–50 AU, spray voltage 4.5 kV, and mass range 100–2000 a.m.u. (maximum resolution 30 000). Structure elucidation of the isolated compounds was carried out on the basis of comparison of their NMR spectral data as well as the HRESI-MS of these compounds with the literature data and differert compound libraries.

Physiological studies on the isolated endophyte

Effect of temperature on the growth of bacteria

The bacterium was incubated statically in NAM at different temperatures (40, 50, 60, and 70°C) for 28 h to determine the optimum temperature for growth.

The role of pH levels on the growth

Nutrient broth media (50 ml in 250 ml flasks) were adjusted to cover the pH range (3, 4, 5, 6, 7, 8, 9, and 10) using 0.1 mol/l NaOH and 0.1 mol/l HCl and buffer solutions. Spore suspension (1 ml) was inoculated in the flasks and shaken for 24 h. Absorbance was read at 600 nm [18].

Influence of different salts

Peptone 0.3, yeast extract 0.15, and glucose 0.5 (g/l) were added to 50 ml of 0.1 mol/l) each of the following salt solutions: $FeCl_3$, Fe_2SO_4, $CuSO_4$, K.phthalate, $ZnSO_4$, EDTA.2Na, Na_2HPO_4, KH_2PO_4, $MgSO_4 0.7H_2O$, NaCl, $CaCl_2$, KCl, KI, $CH_3COONa.3H_2O$, Na_2HPO_4, $NaHCO_3$, Na_2CO_3, and $MnCl_2$. Spore suspension (1 ml) was used to inoculate the medium, which was shaken at 30°C, and the absorbance was read at 600 nm after 28 h.

Significance of different carbon sources on growth production

Carbon sources such as galactose, glucose, raffinose, lactose, mannose, cellobiose, manitol, glycerol, sucrose, and maltose were used in weights equivalent to the carbon amounts in 2 g of glucose and added separately to 50 ml of medium that contained the following (g/l): yeast extract 0.5, KH_2PO_4 0.1, and $MgSO_4.7H_2O$ 0.5. The media were inoculated with 1 ml of spore suspension, shaken for 28 h, and absorbance read at 600 nm.

Results and discussion

Isolation and antimicrobial potential of the marine bacteria

On the basis of the distinct colony morphology of shape, size, and color, 11 endophytic bacteria were isolated from seaweeds, soft corals, and sponge (Table 1). The antagonistic potential of the metabolites in the total extract (300 μg/disc) was tested against six pathogenic organisms (Table 2). Among the 11 isolated endophytes, it is evident that A4 was the most active against the most tested microorganism. Depending on this outcome, A4 was chosen for further biological assessments.

Table 1 The codes of bacterial isolates from marine organisms

Sources	Species	Isolate code
Seaweeds	*Padina pavonica* (brown alga)	A1, A2, A3
	Cystoseira myrica (brown alga)	A4
	Acanthophora dendroides (red alga)	A5
	Sargassum sabrepandum (brown alga)	A6
Soft coral	*Nephthea molle* spp.	C1, C2, C3
Sponge	*Hymedesmia* spp.	S1, S2

Table 2 The antimicrobial screening of the isolated extract (300 μg/disc)

	Test organisms					
Bacterial codes	*Bacillus subtilis*	*Staphylococcus aureus*	*Sarcina maxima*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albicans*
Standard antibiotics						
AM	NI	NI	NI	NI	NI	NI
AX	10.0 ± 0	7.0 ± 0	10 ± 0	7.0 ± 0	7.0 ± 0	7.0 ± 0
E	20.0 ± 0	17.0 ± 0	20.0 ± 0	17.0 ± 0	15.0 ± 0	17.0 ± 0
A1	7.3 ± 0.58	NI	NI	7.3 ± 0.58	NI	7.3 ± 0.58
A2	NI	NI	7.0 ± 1.0	NI	8.3 ± 0.58	NI
A3	8.3 ± 0.58	7.0 ± 1.0	NI	10.0 ± 1.0	7.0 ± 0	7.0 ± 1
A4	9.7 ± 0.58	14.3 ± 0.58	20.3 ± 0.58	8.0 ± 0	10.7 ± 0.58	8.3 ± 0.58
A5	8.0 ± 1.0	7.0 ± 0	7.3 ± 0.58	12.3 ± 0.58	11.0 ± 1.0	NI
A6	14.0 ± 1.0	11 ± 1.0	18.3 ± 0.58	NI	12.0 ± 0	NI
C1	8.0 ± 1.0	NI	NI	NI	NI	NI
C2	7.3 ± 0.58	NI	NI	NI	7.3 ± 0.58	NI
C3	7.0 ± 0	NI	NI	NI	NI	NI
S1	NI	NI	NI	NI	NI	NI
S2	NI	NI	NI	NI	10.3 ± 0.58	NI

AM, ampicillin 10 mcg; AX, amoxicillin 25 mcg; E, erythromycin 15 mcg; NI, no inhibition.

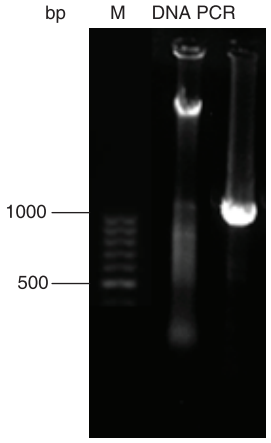
Isolation, identification, and phylogenetic analysis

Only one isolate, A4, was selected as a promising producer of bioactive compounds. Molecular identification of this isolate based on 16S rDNA sequencing was carried out. A fraction of the PCR mixture was examined using agarose gel electrophoresis (Fig. 1). According to sequencing similarities and multiple alignment, the isolate A4 was found to be closely related to *E. cloacae* strain GH1 (ac: JF261136.1) with a 98% identity. The phylogenetic tree was displayed using the TreeView program (Fig. 2) [19].

Influence of culture composition on the production of bioactive metabolites

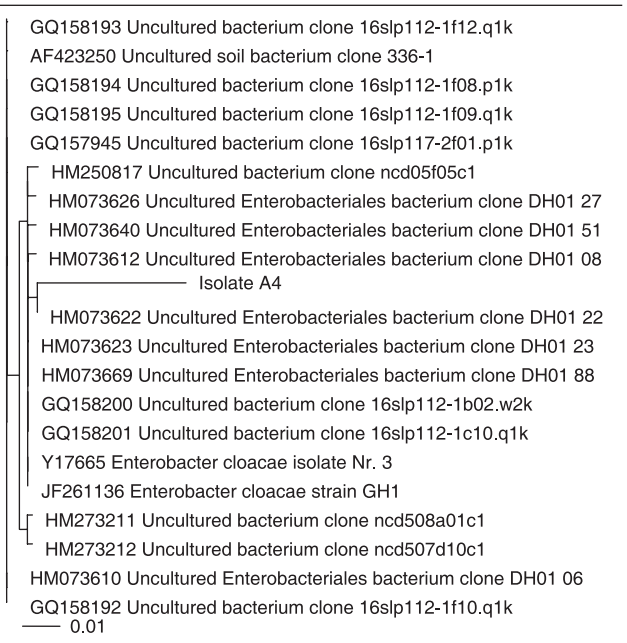
To detect the role of cultivation media and salinity on the production of active metabolites, five media were examined. It was clearly obvious that media I, which was mainly composed of 10 g glucose in 50% seawater, was the most favorable for the production of bioactive compounds and showed inhibition against most pathogens (Figs. 3–7). In contrast, the decrease in glucose concentration in media II and III led to weak activity (Figs. 4 and 7) or a complete loss of activity (Figs. 3 and 5). The presented data proved that *S. maxima* was the most sensitive strain to the metabolites produced in the fermentation medium (I) by *E. cloacae* at all concentrations used (Fig. 4), followed by *S. aureus* (Fig. 5). The data also showed sensitivity of *B. subtilis* to the metabolites in media IV (Fig. 3). These results indicated the significance of carbon source concentrations and salinity in the production of bioactive compounds. To the best of our knowledge the biological activity of *E. cloacae* has not been investigated before. Seventy-five marine bacterial strains from sponges were isolated to evaluate the antimicrobial potential; 21% of the bacterial strains were found to be antibiotics

Figure 1



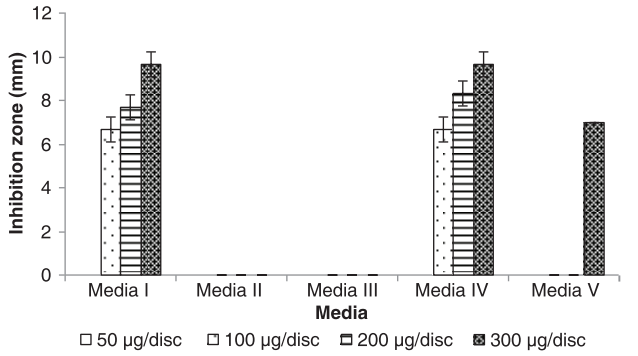
Agarose gel electrophoresis.

Figure 2



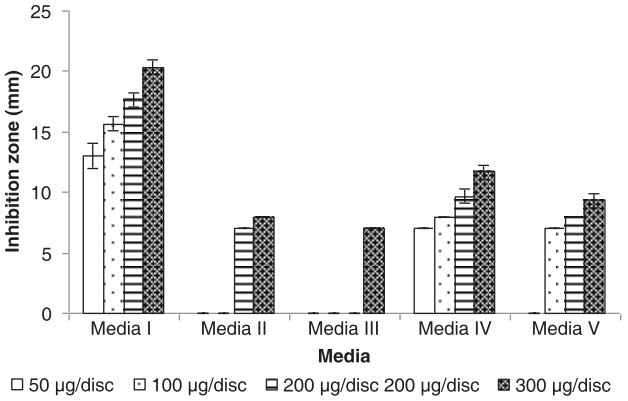
The phylogenetic tree based on PCR product sequencing of DNA isolated from the endophytic bacterium A4.

Figure 3



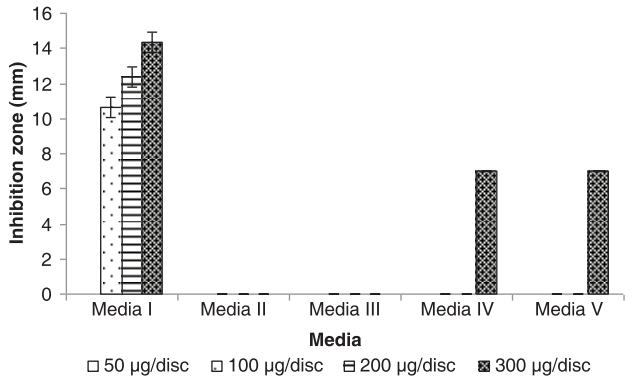
Antimicrobial activity of *Enterobacter cloacae* extract on *Bacillus subtilis*.

Figure 4



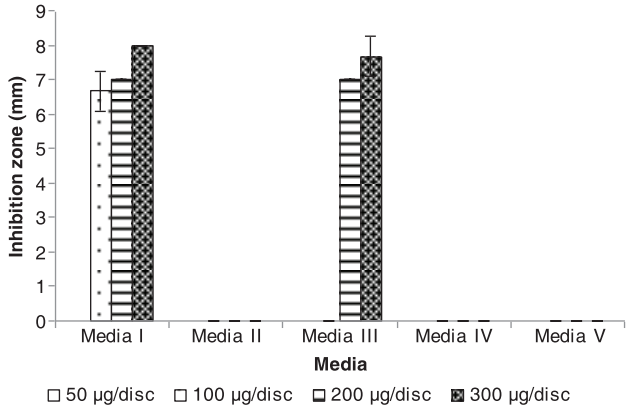
The effect of different concentrations of *Enterobacter cloacae* metabolites on *Sarcina maxima*.

Figure 5



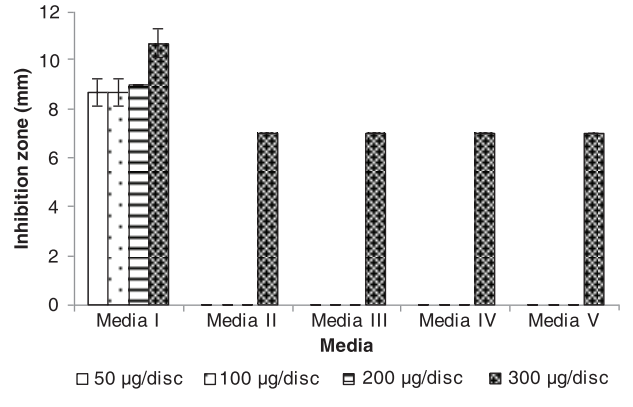
Bioactive potential of *Enterobacter cloacae* metabolites on *Staphylococcus aureus*.

Figure 6



Antagonistic activity of *Enterobacter cloacae* extract against *Escherichia coli*.

Figure 7



Antimicrobial activity of *Enterobacter cloacae* crude extract against *Pseudomonas aeruginosa*.

producers and their activities ranged from broad spectral to species specific [20]. In addition, 12 isolates from Brazilian sponges presented antimicrobial activities against Bacillus spp., *Virgibacillus* spp., *Pseudovibrio* spp., *Pseudomonas* spp., and *Stenotrophomonas* spp. [21]. An antibiotic producer *Pseudomonas* spp., from the Persian Gulf, was also investigated [22].

Antioxidant activity

The results in Table 3 showed good antioxidant activity for the endophyte extract at a concentration of 2 mg/ml (IC_{50} was obtained at 1.266 mg/ml). In contrast, the other concentrations revealed a weak activity. To our knowledge this is the first report on the antioxidant activity of *E. cloacae* metabolites. The antioxidant activity of the metabolites produced by the marine bacteria *Virgibacillus* spp. associated with the sponge *Callyspongia diffusa* was studied [23]. This research revealed the potential of marine sponge-associated bacterial bioactive compounds in scavenging free radicals in vitro.

Our results on the antioxidants from endophytic bacteria were supported by those of Takao *et al.* [24], who isolated 112 marine bacterial strains with antioxidant activity. Further, Krishna *et al.* [25] focused on the antioxidant potential of marine *B. subtilis* (MTCC no. 10619). They concluded that the production of catalase enzyme, nonenzymatic parameters, and ascorbic acid was responsible for the activity.

Antiviral activity

This extract was evaluated for in-vitro antiviral activity. The present findings showed that all the embryos of the positive controls (inoculation of the virus without the extract) had died and the allantoic fluid of each was positive in the hemagglutination assay, whereas the inoculation of the bacterium extract with H5N1 and NDV viruses inhibited the virus effects (Table 4). A marked decrease in hemagglutination and in the number of dead embryos was observed. The results obviously showed that the bacterium was effective against both respiratory RNA virus (H5N1) and NDV. The resistance of viral pathogens to existing treatment drugs has increased the demand for new medicines. The screening of natural products derived from marine species for antiviral activity has yielded a considerable number of active crude aqueous and organic solvent extracts. Today, over 40 compounds are commercially available in pharmacological markets, including alternative antiviral medicines [26]. A team of research scientists evaluated the antiviral and immunoregulatory effect of a novel exopolysaccharide (EPS-1) from a marine thermotolerant *Bacillus licheniformis* and found that EPS-1 treatment impaired HSV-2 replication in human peripheral blood mononuclear cells [27]. In contrast, marine bacteria with antiviral properties have been isolated and tentatively classified as

Moraxella. They concluded that the antiviral capacity of the isolated microorganisms was highly specific for poliovirus [28].

Table 3 Antioxidant activity of *Enterobacter cloacae* extracts using DPPH assay

Concentrations (mg/ml)	Antioxidant activity (%)
0.5	32.17
1.00	43.65
1.50	59.24
2.00	72.19
2.50	71.93

DPPH, 1,1-diphenyl-2-picryl-hydrazil.

Table 4 Antiviral activity of the endophytic bacterium metabolites

Virus	μg/ml	NDE	Positive HA
Control	—	5/5	5.0
NDV	3.2	1/5	1.0
H5N1	3.8	2/5	2.0

HA, hemagglutination assay; NDE, number of died eggs; NDV, newcastle disease virus.

Fractionation and identification of the active compounds

The literature review revealed no information on the chemistry of the *Enterobacter* spp. extract. The medium pressure liquid chromatography column, with silica gel 60, yielded 120 fractions. Thin layer chromatography monitoring of different fractions was carried out using hexane : ethyl acetate (S1 8 : 2, S2 7 : 3, S3 5 : 5), and spots were visualized using ultraviolet and *P*-anisaldehyde/H_2SO_4 spray reagent. Eight major fractions were collected (F1–F8) (Table 5). LC/HRESI-MS of the collected fractions was carried out showing the chemical pattern of each fraction. Eight compounds out of 15 were identified by comparing the MS/MS fragmentation pattern with the library database. Data showed that around 18 compounds were repeated in different fractions.

The identified compounds were found to be localized in fractions F6, F7, and F8. It was clear in (Table 6) that the following identified compounds

Table 5 Liquid chromatography/mass spectrometry data of *Enterobacter* fractions with the suspected formula and suggested identified compounds

Fraction numbers	Numbers	t_R	MW	Compound formula	Identification
F1	1	19.08	425.2144	$C_{18}H_{29}O_6N_6$	No hits
	2	21.87	429.2396	$C_{24}H_{33}O_5N_2$	No hits
	3	23.96	431.1785	$C_{17}H_{27}O_9N_4$	No hits
	4	26.05	663.4543	$C_{33}H_{59}O_6N_8$	No hits
F2	1	16.51	337.1049	$C_{16}H_{13}O_3N_6$	No hits
	2	19.12	425.2144	$C_{18}H_{29}O_6N_6$	No hits
	3	21.88	429.2396	$C_{24}H_{33}O_5N_2$	No hits
	4	23.98	431.1785	$C_{17}H_{27}O_9N_4$	No hits
	5	26.04	663.4543	$C_{33}H_{59}O_6N_8$	No hits
F3	1	13.19	219.1744	$C_{15}H_{23}O$	No hits
	2	21.74	804.5466	$C_{42}H_{78}O_{13}N$	No hits
F4	1	13.73	323.1942	$C_{17}H_{27}O_4N_2$	No hits
	2	19.11	425.2145	$C_{18}H_{29}O_6N_6$	No hits
	3	21.75	429.2396	$C_{24}H_{33}O_5N_2$	No hits
	4	26.19	663.4543	$C_{33}H_{59}O_6N_8$	No hits
F5	1	3.68	323.151	$C_{13}H_{23}O_7S$	No hits
	2	16.79	437.1933	$C_{22}H_{25}O_4N_6$	No hits
	3	21.75	429.2396	$C_{24}H_{33}O_5N_2$	No hits
	4	26.19	663.4543	$C_{33}H_{59}O_6N_8$	No hits
F6	1	9.20	211.1442	$C_{11}H_{19}O_2N_2$	Cyclo(leucylprolyl)
	2	12.92	227.1754	$C_{12}H_{23}O_2N_2$	l-Leucine anhydride
	3	19.12	425.2144	$C_{18}H_{29}O_6N_6$	No hits
	4	21.78	429.2396	$C_{24}H_{33}O_5N_2$	No hits
	5	26.10	663.4543	$C_{33}H_{59}O_6N_8$	No hits
F7	1	7.15	197.1286	$C_{10}H_{17}O_2N_2$	Cyclo(prolylvalyl)
	2	10.05	245.1284	$C_{14}H_{17}O_2N_2$	Cyclo(l-phe-l-pro)
	3	12.97	227.1754	$C_{12}H_{23}O_2N_2$	l-Leucine anhydride
	4	19.15	425.2144	$C_{18}H_{29}O_6N_6$	No hits
	5	21.74	429.2396	$C_{24}H_{33}O_5N_2$	No hits
F8	1	6.74	261.1234	$C_{14}H_{17}O_3N_2$	Cyclo(tyrosyl-prolyl)
	2	7.54	227.1390	$C_{11}H_{19}O_3N_2$	Cyclo(d-hypro-l-leu)
	3	8.44	261.1235	$C_{14}H_{17}O_3N_2$	d,d-4-Hydroxyprolyl-phenylalanyl
	4	13.58	243.1703	$C_{12}H_{23}O_3N_2$	No hits

MW, molecular weight; t_R, retention time.

Table 6 Antimicrobial activity of eight fractions (150 mg/disc) of *Enterobacter* spp. against some pathogenic microorganisms

	Inhibition zone (mm)							
Test organisms	F1	F2	F3	F4	F5	F6	F7	F8
Bacillus subtilis	6.0 ± 0.0	8.3 ± 0.58	8.0 ± 0.0	NI	NI	NI	NI	10.7 ± 0.58
Staphylococcus aureus	8.3 ± 0.58	9.0 ± 1.0	8.3 ± 0.58	7.0 ± 0.0	7.3 ± 0.58	7.3 ± 0.58	NI	8.0 ± 0.0
Sarcina lutea	NI	NI	NI	NI	NI	NI	8.0 ± 0.0	12.7 ± 0.58
Escherichia coli	7.0 ± 0.0	8.0 ± 0.0	8.7 ± 0.58	9.7 ± 0.58	8.3 ± 0.58	7.3 ± 0.58	NI	NI
Pseudomonas aeruginosa	0.0 ± 0.0	7.3 ± 0.58	7.3 ± 0.58	NI	NI	NI	NI	8.0 ± 0
Candida spp.	0.0 ± 0.0	8.0 ± 0.58	8.3 ± 0.58	NI	NI	NI	NI	9.0 ± 0.0

NI, no inhibition.

in fractions 6 and 7 had weak activities against the examined microorganisms: cyclo (leucylprolyl), cyclo (L-phe-L-pro), cyclo (prolylvalyl), cyclo (L-phe-L-pro), and L-leucine anhydride. In contrast, a moderate activity was seen for cyclo (tyrosyl-prolyl), cyclo (D-hypro-L-leu), and D,D-4-hydroxyprolyl-phenylalanyl in F8; these compounds were responsible for the antipathogenic activity against most microbes. The unidentified compounds revealed a range of molecular formulae, but the presence of nitrogen was a common feature among most of them, which could lead to the discovery of new diketopiperazine or other peptide derivatives as published before [29].

Physiological studies

In this study we tried to ascertain the appropriate conditions for E. cloacae growth. It was obvious that the cells were resistant to very low and high temperatures (20 and 50°C) and it was noticed that the favorable conditions for maximum growth of the isolated bacterium was 30°C (Table 7) and pH 7 (Fig. 8). The alkaline media at pH 8 and 9 also aided in good germination of the microorganism. Reviewing the literature, a study on the interactive effect of temperature and pH on EPS production by *Enterobacter* spp. A47 was reported. The results revealed the ability of *Enterobacter* spp. A47 to synthesize different heteropolysaccharides as a function of pH and temperature, and this synthesis was stable for wide pH and temperature ranges [30]. In contrast, the optimum temperature for the growth of the facultative anaerobe *Enterobacter aerogenes* was about 40°C at pH 7.0 [31].

Endophyte growth was evaluated in media supplemented with different carbon sources and salts. The results in Table 8 show that maltose was the most favorable nutrient for the organism, followed by mannose (96.8%) and raffinose (89.6%). The addition of potassium salts (KI and KCl) to cultivation media gave the most favorable results, followed by CaCl2 (Table 9). The growth responses of the marine organisms when various groups of salts were omitted in turn from artificial seawater were evaluated. It was evident that removing either Na^+ or K^+ salts completely hampered growth in all cases [32]. It

Table 7 Effect of different temperatures on the growth of *Enterobacter cloacae*

Temperatures (°C)	Growth
10	–
20	+
30	+++
40	++
50	+
60	–

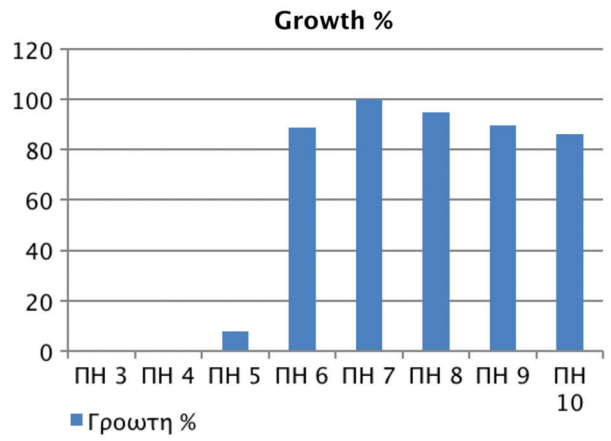
Table 8 Effect of different carbon sources on the growth of *Enterobacter cloacae*

Carbon sources	Growth (%)
None	45.7
Galactose	52.7
Glucose	37.9
Raffinose	89.6
Lactose	28.2
Mannose	96.8
Cellobiose	73.4
Manitol	45.9
Glycerol	—
Sucrose	70.9
Maltose	100

Table 9 The role of different salts on the growth of *Enterobacter cloacae*

Salts	pH	Growth (%)
None	6.8	59.6
$FeCl_3$	1.4	—
Fe_2SO_4	3.55	26.6
$CuSO_4$	3.51	—
K.phthalate	4.13	1.0
$ZnSO_4$	4.81	—
EDTA.2Na	4.88	—
Na_2HPO_4	5.03	0.5
KH_2PO_4	5.4	1.45
$MgSO_4.7H_2O$	5.64	42.6
NaCl	5.7	38.8
$CaCl_2$	5.77	56.6
KCl	5.91	59.5
KI	6.04	100
$CH_3COONa.3H_2O$	6.0	0.3
Na_2HPO_4	8.38	51.6
$NaHCO_3$	9.13	4.5
Na_2CO_3	10.67	6.6
$MnCl_2$	5.1	0.6

Figure 8



Effect of pH on the growth of *Enterobacter cloacae* on using PBS.

was reported that the optimal temperature for the growth of *Enterobacter* spp. is between 30 and 37°C and the microbe has a fermentative type of metabolism. D-Glucose and other carbohydrates are catabolized with the production of acid and gas. The carbohydrates that are fermented by *Enterobacter* spp. include mostly cellobiose, L-arabinose, D-mannitol, maltose, D-mannose, trehalose, and salicin [33].

Conclusion

This article focused on the bioactive potential of the metabolites produced by E. cloacae, which revealed a promising inhibition against pathogenic bacteria and H5N1 and NDV viruses. Antioxidant activity was detected in the total extract of the marine bacterium from which diketopiperazines derivatives were isolated. This study explores the possibility for the discovery and use of new drugs from natural sources.

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Conflicts of interest

There are no conflicts of interest.

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