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GC-MS Analysis of Bioactive Compounds in Methanolic Extract of Marine *Enterobacter Cloacae*

Mariam ADRA[1*], Badr ALALI[2] and Ahmad KARA ALI[3]

[1,2]Department of Marine biology, Higher Institute of Marine Research, Tishreen University, Lattakia, Syria
[3]Department of Marine Chemistry, Higher Institute of Marine Research, Tishreen University, Lattakia, Syria

[*]*Corresponding Author: adramariam94@gmail.com*

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Abstract— Antimicrobial substances are so widespread that they are likely to play an important therapeutic role. Many researches showed that microorganisms produce important biochemical compounds, which were used in different domains (medical, pharmaceutical, industrial). The present study aimed to isolate a marine bacterium and study the bioactivity of its methanolic crude extracts. Marine water samples were collected for the isolation of bacteria based on their growth density and then identified depended on morphological, microscopic characterization and biochemical tests. The antibiotics sensitivity was estimated by DL-96II Auto Microbial ID/AST System, While the effectiveness of the methanolic extract was determined by agar- well diffusion method. The compounds in the organic extract of the isolated bacteria were identified by Gas chromatography – mass spectrometry (GC-MS), and their bioactivity was studied. The results showed that these bacteria is *Enterobacter cloacae* and showed resistance to six from twenty antibiotics. The bioactivity of extract was higher against *Pseudomonas aeruginosa* and *Escherichia coli* than *Staphylococcus aureus.* The chemical analysis of the organic extract showed that eighteen chemical compounds have medical and pharmaceutical importance.

Keywords—*Marine Enterobacter cloacae, Antibiotics, Bioactivity, GC-MS analysis.*

I. INTRODUCTION

The secondary metabolites produced by marine organisms have more novel and unique structures from terrestrial organisms, which is due to the specific conditions of marine environment and high biodiversity, their bioactivity are high [1],[2],[3].

Further more, most of new researches about marine natural products in marine animal indicate that their source is associated to microorganisms [4],[5],[6].

Competition among microbes for the space and the nutrient in marine environment induces marine microorganisms to produce many natural products, which have a medical and industrial values [5]. Because of the specific role of the symbiotic bacteria in their special hosts, many antimicrobial, antifouling substances have been found among the symbiotic of bacteria [7],[8],[9]. It is suggested that the primary role of these antibiotic substances could be related to ecological competition. If this were true, we would expect the antibiotic producing bacteria associated with some particular hosts to be proportionally higher than others. However, few investigations have been conducted to study and compare the antibiotic activities of marine bacteria isolated from different origins [7],[10],[11]. For example; *Enterobacter* genus belongs to the Enterobacteriaceae family. This genus includes many of species *E. cloacae*, *E. aerogenes*, *E. gergoviae* and *E. agglomerans*. *Enterobacter* cells are gram-negative cells and facultative anaerobes. This genus is ubiquitous; its presence in the intestinal tract of animals results in its wide distribution in soil, water and sewage [12],[13],[14].

This study aimed to isolate marine *Enterobacter cloacae* and determine the chemical composition of the methanolic extract of marine *Enterobacter cloacae* and its bioactivity.

II. METHODOLOGY

Sampling site:

Sea water samples were collected from of the Mediterranean coast at Lattakia Port site, Lattakia, Syria (35°30'51.6"N 35°46'08.9"E), in May 2019 (Figure 1).The samples were collected in sterile glass bottles and brought to laboratory in the ice box and processed within one hour from collect.

Figure 1: Sampling station in the Lattakia port site.

Serial dilution method:

1 ml from the samples were labeled according to their location, was added 9 ml of saline water, and serially diluted. Each dilution was used to isolate the bacteria by using nutrient broth medium.

Isolate bacteria:

The nutrient broth enriched samples were spreaded on nutrient agar medium and incubated for 24 and 48 hours at 32 °C in bacteriological incubator.

Identification of isolated marine bacteria:

The well growth colonies of marine bacteria were isolated and purred. Then, we choised one of them for study their colonies and cells characterization including; the morphological, microscopic and many of biochemical tests (sugar fermentation, TSI, IMVIC, gram strain, catalase and oxidase) (table 1) and their sensitivity to antibiotics (table 2).

Sub culturing and preservation:

Studied isolates were re-streaked on the marine agar plates and incubated for 24h at 32 °C and after 24 h the colonies were checked for purity by gram straining and streaked on slope of marine agar, after 24h they kept at 4 °C for others tests.

Preparation of bacterial suspension:

For prepare bacterial suspension, first, 24 hours prior testing, bacterial transferring from stored culture to nutrient broth (Merck, Germany) and incubated for 24 h at 32 °C, then it was streaked on nutrient agar (Merck, Germany) and incubated for 24 h at 32 °C. After that, the colonies grown were removed with normal saline solution for obtain concentrated bacterial suspension. Then the amount of the bacterial suspension was poured into tubes containing sterile saline. The turbidity was measured with a spectrophotometer at wavelength of 630 nm for adjusted the optic density at 0.5 which equal to 1×10^{8}CFU / ml.

Sensitivity of isolated marine bacteria to antibiotics:

Sensitivity of *Enterobacter cloacae* to antibiotics was estimated by DL-96II Auto Microbial ID/AST System and the results were recorded.

Preparation of isolated marine bacteria extract:

The liquid culture aged 24h was centrifuged with 10,000 rpm for 10 minute and the supernatant was separated from the cells. Methanolic extract of bacterial cells was obtained at (V:V) and soaking for 24 hours at room temperature. The mix was placed in the ultra-sonic device for an hour. The methanol solvent was separated from the mix using the separation funnel and the residual solvent was evaporated using the liquid nitrogen pump. The concentrated organic extract was kept at -20 °C until chemical analysis by GC-MS.

Antibacterial activity test of organic extract of isolated marine bacteria:

The Antibacterial activity of the organic extract of *E. cloacae* was studied by agar- well diffusion method.

Muller Hinton agar plate was prepared. The liquid cultures of four bacterial isolates pathogens (*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumonia*) grown for 24h at 37 C, were uniformly swabbed on the surface of Muller Hinton agar plates using sterile cotton swab. Four wells of 6mm were made with sterile cork borer on the seeded plate. Around 30 µl of organic extract was added to the well aseptically. The plates were incubated for 24h at 37°C and the zone of inhibition was observed and recorded (in mm).

Chemical analysis of organic extract by (GC-MS):

The organic extract was analyzed by gas chromatography–mass spectrometry (GC–MS). GC analysis was carried out using an a gilent technologies HP-6890 with capillary column (30m × 320 µm i.d., film thickness 0.25 µm) and connected to a MS was used, the column temperature was programmed from 70 to 280 at rate 4 °C / min. The carrier gas was helium with a flow rate of 1.2 ml/min.

III. RESULTS AND DISCUSSION

Identification of Enterobacter spp. :

Identification of *Enterobacter spp.* depended on morphological, microscopic characterization and biochemical tests. The results showed that marine bacteria isolate was gram negative rod shape (Figure 2). According to the references Bergey's manual of determinatie bacteriology (1994) and Bergey's manual of systematic bacterialogy (2004) [15],[16].

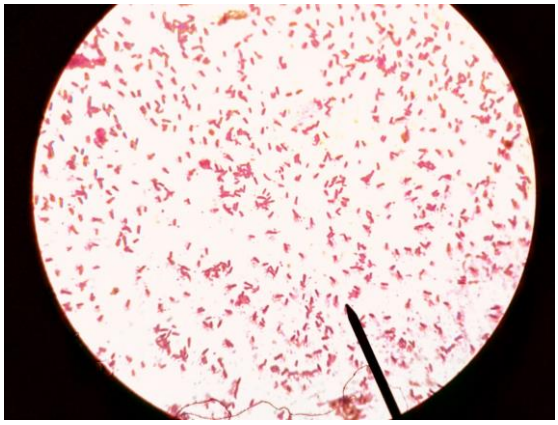

Figure 2: Microscopic image of Enterobacter cloacae (Gram Stain).

The table (1) showed that this isolate was positive for each of the following bio-chemical tests; arabinose, melibiose, glucose, rhamnose, Voges–Proskauer, methyl red, β-galactosidase, raffinose, malonate, sucrose, ornithine, arginine, citrate, cellobiose, malate, lactose. While it was negative for the rest of bio-chemical tests; sorbitol , H_2S production, urease, inositol, indole production, gelatinase, adonitol, lysine, esculin hydrolysis, α-methyl-D-glucoside, salicin, oxidase. These characteristics refer to this strain is *E. cloacae* according to the Bergey's manual of determinatie bacteriology (1994) and Bergey's manual of systematic bacterialogy (2004) [15],[16].

Table 1. Bio-chemical tests of Enterobacter cloacae

No.	Bio-chemical test	Code	Result
1.	Arabinose	ARA	Positive
2.	Melibiose	MEL	Positive
3.	Glucose	GLU	Positive
4.	Rhamnose	RHA	Positive
5.	Inositol	INO	Negative
6.	Sorbitol	SOR	\Negative
7.	Raffinose	RAF	Positive
8.	Adonitol	ADO	Negative
9.	Malonate	MAL	Positive
10.	Sucrose	SUC	Positive
11.	Malate	MLT	Positive
12.	Lactose	LAC	Positive
13.	Methyl red	MR	Negative
14.	H2S production	H2S	Negative
15.	Urease	URE	Negative
16.	Indole production	IND	Negative
17.	Voges–Proskauer	VP	Positive
18.	β-galactosidase	ONPG	Positive
19.	Gelatinase	GEL	Negative
20.	Arginine	ARG	Positive
21.	Lysine	LYS	Negative
22.	Ornithine	ORN	Positive
23.	Esculin hydrolysis	ESC	Negative
24.	Citrate	CIT	Positive
25.	Cellobiose	CEL	Positive
26.	α-methyl-D-glucoside	MDG	Negative
27.	Salicin	SAL	Negative
28.	Oxidase	OXE	Negative

Antibiotics sensitivity of Enterobacter cloacae:

Antibiotics sensitivity of *E.cloacae*was carried out by DL-96II Auto Microbial ID/AST System. The results showed that this marine bacterial isolate was sensitive for the most Antibiotics used like; Gentamicin, Cefepime, Ceftriaxone, Cefoperazone / Sulbactam, Piperacillin/ tazobactam, Ticarcillin/Clavulanin acid, Moropenem, Imipenem, Amikacin, Trimethoprim/ sulfamethoxazole, Levofloxacin, Ciprofloxacin, Ceftazidime, Minocycline. While it showed resistance to 5 antibiotics; Cefazolin, Ampicillin, Cefuroxime sodium, Cefoxitin , Chloramphenicol (Table 2) .

Table 2. Antibiotic sensitivity of Enterobacter cloacae:

Antibiotic name	MIC(ug/ml)	Sensitivity
Cefazolin	>4	R
Gentamicin	≤1	S
Ampicillin	>16	R
Cefuroxime sodium		R
Cefepime	≤2	S
Cefoxitin	>16	R
Ceftriaxone	≤1	S
Cefoperazone/sulbactam	≤2/1	S
Piperacillin/tazobactam	≤4/4	S
Ticarcillin/clavulanin acid	≤4/2	S
Sulbactum		R

Moropenem	≤1	S
Imipenem	≤1	S
Amikacin	≤4	S
Trimethoprim/sulfamethoxazole	≤0.5/9.5	S
Levofloxacin	=1	S
Ciprofloxacin	=0.5	S
Ceftazidime	≤4	S
Chloramphenicol	>16	R
Minocycline	≤4	S

S=sensible, I=Intermediate, R=Resistant

Antibacterial activity of organic extract of E. cloacae:

The result showed a higher effect of methanol extract against *Pseudomonas aeruginosa* and *Escherichia coli* compared with the different antibiotics tested in this article. While this activity was low against *Staphylococcus aureus*, and it was nearly with other antibiotics against *Klebsiella pneumonia* (Table 3)

Table 3. Zone of inhibition of methanol extract of E. cloacae against bacterial pathogens by agar well diffusion method

No.	Bacterial pathogens	Methanolic extract (mm)	Co-Trimaxazole	Gentamycin	Amikacin	Bacitracin	Oxy-Tetrcycline	Ampicillin
1	*Staphylococcus aureus*	12	16	15	24	-	-	23
2	*Escherichia coli*	22	9	15	23	-	-	6
3	*Klebsiella pneumoniae*	18	17	20	22	-	16	6
4	*Pseudomonas aeruginosa*	20	16	11	15	11	18	7

Chemical Analysis of organic extract of *E. cloacae* by (GC-MS):

(Fig. 3) shows the presence of various bioactive compounds with different retention times (RT). (Table 4) summarizes the nature of the some identified compounds and their biological activities. It indicates to the most of important chemical compounds identified.

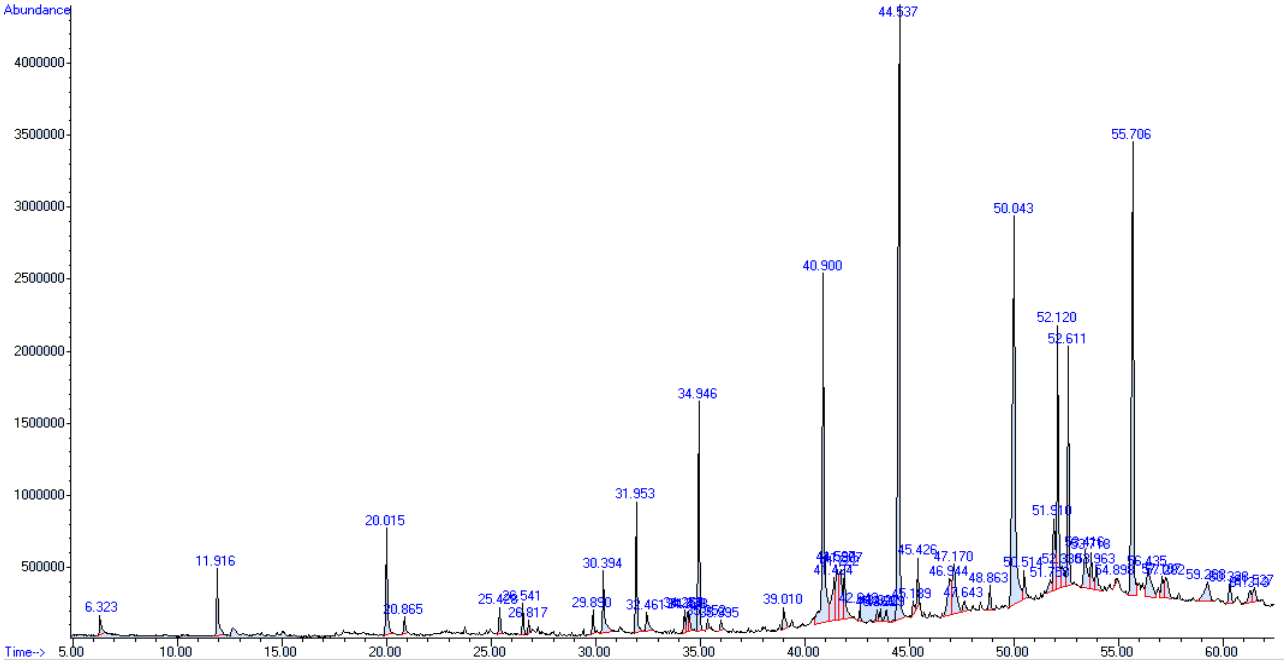

Figure 3: GC–MS chromatogram of bio-active compounds of *Enterobacter cloacae* methanolic extract.

Table 4. The most important of identified compounds in methanolic extract of Enterobacter cloacae by GC-MS analysis and their biological activity

No.	Compound Name	Molecular Formula	Compound Nature	Bioactivity
1.	Tetradecanoic acid	$C_{14}H_{28}O_2$	Fatty acid	Anti-bacterial, anti-cancer, antioxidant, decrease renal damage, hypercholesterolemic, lipid anchor of biomembrane and Nematicide [17].
2.	Hexadecenoic acid, Z-11-	$C_{16}H_{30}O_2$	Fatty acid	Pesticide and antibiotic [18].
3.	4-(3,4-Methylenedioxyphenyl)-2-butanone	$C_{11}H_{12}O_3$	Aromatic ketone	Antineoplastic, drugs for dermatological problem [19].
4.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	Palmitic Acid	Anti-inflammatory [20]. Antioxidant, hypocholesterolemic, nematicide, pesticide, anti an ogenic, hemolytic, antipsychotic and 5-Alpha reductase inhibitor [21]. Potent mosquito larvicide[22].
5.	7H-Furo[3,2-g][1]benzopyran-7-one,4-methoxy-(Bergaptan)	$C_{12}H_8O_4$	Ketone	Anti-inflammatory , analgesic , strong cytotoxic activities on humman leukemia and chemopreventive effects on hepatitis and skin tumorsm [23].
6.	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	Fatty acid	Anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematicide, insectifuge, antihistaminic, antieczemic, antiacne, 5-Alpha reductase inhibitor, antiandrogenic, antiarthritic and anticoronary [24].
7.	Oleic Acid	$C_{18}H_{34}O_2$	Fatty acid	Antibacterial [25]. Antiinflammatory, antiandrogenic cancer preventive, dermatitigenic hypocholesterolemic, 5-Alpha reductase inhibitor, anemiagenic insectifuge and Flavor [26].
8.	4,4'-Dimethoxydiphenylamine	$C_{14}H_{15}NO_2$	Aldehyde	Used in the synthesis of amine electron donating groups in organic donor-bridge-acceptor dyads in Dye Sensitized Solar Cells (DSSC) [27]. Chemical additive for cured rubber, it is highly toxic and may potentially induce chromosome abberation. [28].
9.	Ditetradecyl ether; Tetradecyl ether	$C_{28}H_{58}O$	Ketone	Antimicrobial and anticorrosion activities [29].
10	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	Amino compound	Hemolytic, pesticide and flavour, antioxidant [30].
11	1,2-Benzenedicarboxylic acid, Dioctyl ester	$C_{24}H_{38}O_4$	Fatty acid	Antimicrobial and anti- fouling [31].
12	Hexacosane	$C_{26}H_{54}$	Hidrocarbon	Antimicrobial and antifungal agents [32].
13	Retinoic acid, methyl ester (Beta-Progesterone)	$C_{21}H_{30}O_2$	Ester	Anti-inflammatory, normalize blood clotting and prevent from endometrial cancer [17].
14	Heptacosane	$C_{27}H_{56}$	Hidrocarbon	Antioxidant activity [33].
15	Nonacosane	$C_{29}H_{60}$	Hidrocarbon	Antibacterial activity [34].
16	Heneicosane	$C_{21}H_{44}$	Hidrocarbon	Antibacterial, antimicrobial, antiasthmatics, urine acidifiers and antimicrobial [35].
17	Vitamin E	$C_{29}H_{50}O_2$	Vitamin E	Antiageing, analgesic, antidiabetic, anti-inflammatory, antioxidant, antidermatitic, antileukemic, antitumor, anticancer, hepatoprotective, hypocholesterolemic, antiulcerogenic, vasodilator, antispasmodic, antibronchitic and anticoronary [36].
18	Octacosanoic acid	$C_{28}H_{56}O_2$	Fatty acid	Antifungal, antitumour and antibacterial activity [37].

The GC-MS analysis of *Enterobacter cloacae* methanolic extract revealed the presence of 18 chemical compounds. These compounds possess many biological properties. Examples mentioned in this article include the potent cytotoxic Bergaptan activity on human leukemia and skin tumors [23]. Which may be a promising therapeutic agent for cancer. As well as, its chemopreventive effects on hepatitis [23]. Which could be helpful in preventing the initiation stage of carcinogenesis. Vitamin E was detected also in methanolic extract of *E. cloacae* has the properties of Antiageing, analgesic, antidiabetic, anti-inflammatory, antioxidant, antidermatitic, antileukemic, antitumor, anticancer, hepatoprotective, hypocholesterolemic, antiulcerogenic, vasodilator, antispasmodic, antibronchitic and anticoronary [29].

Oleic acid is another compound present, which can act as antioxidant and anti-inflammatory property. This acid may hinder the progression of adrenoleukodystrophy (ALD), a fatal disease that affects in the brain and adrenal glands [35],[38]. It may be responsible for the hypotensive (blood pressure reducing) effects of olive oil [32],[39], antiandrogenic, cancer preventive, dermatitigenic, hypocholesterolemic, 5-alpha reductase inhibitor and anemiagenic [30].

Discussion

These activities catalyze the continued interest to marine microbial natural products and reflect the need more intensive studies for their chemical, pharmacological and medical properties.

The present compounds in methanolic extract of *E. cloacae* were being used for the pharmacological work. Thus, this type of GC-MS analysis is the first step towards understanding the nature of active compounds in the marine bacteria and this type of study will be helpful for further detailed study. However, isolation of individual chemical constituent and subjecting it to biological activity will definitely give important results. It could be conclude that *E. cloacae* contains various bioactive compounds. So it is recommended as important pharmaceutical organism. However, further studies are needed to undertake its bioactivity and toxicity profile.

IV. Conclusion and Future Scope

In our study as in the previous studies we confirm the presence of compounds with important biological activities extracted from marine bacteria. Thus, marine bacteria are a promising source for many important compounds. Therefore it is suggested to expand biological studies of marine microbial natural products to discover their exceptional biological activities, and extensive investigation of their chemical and pharmacological properties. Which may be important for treating some chronic diseases

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Compliance with Ethical Standards:

Conflict of Interest:

Author1 (Mariam Adra) declares that she has no conflict of interest.

Author2 (Badr Alali)declares that he has no conflict of interest

Author3 (Ahmad Kara Ali) declares that he has no conflict of interest.

Ethical approval:

This article does not contain any studies with human participants or animals performed by any of the authors.

Short description of the contributions made by authors:

MA and BA carried out the biological experiments, MA attended the organic extract, AK analyzed the extract using GC-MS device, MA analyzed the data and revealed her biological properties, MA wrote the manuscript. All authors read and approved the manuscript.

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AUTHORS PROFILE

Mrs. Mariam Adra has obtained B. of Microbiology from Tishreen University in 2016, and is studying MSc of Marine Biology science at the Higher Institute for Marine Research. She works as a science teacher at the secondary level and teaches bacteriology and antibiotics at the university. Her main research work focuses on bacteria. she has 5 years of teaching experience and 3 years of research experience.

