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Evaluation of the Activity of the Diatom *Navicula Incerta* Cultivated in Different Environments Against Several Species of Pathogenic Bacteria

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Abstract

Diatoms are considered a potentially new and valuable source of biologically active compounds including those with antimicrobial properties; so, this study was conducted to evaluate the antibacterial activity of *Navicula incerta*. The diatom was isolated from the salt water of Sawa Lake, southern of Iraq, it was cultivated in salt water then was adapted and cultivated in freshwater environment; the harvested and dried biomass was extracted, and the antibacterial activity of each extract was evaluated against several species of pathogenic bacteria. The chemical constituents of the extracts were also analyzed using Gas chromatography/Mass spectrometry technique. Generally, the result showed that fresh water extract of *N. incerta* has higher antibacterial activity than salt water extract; Furthermore, the diatom extract inhibited the growth of *Staphylococcus aureus* strain that was resistant to five different antibiotics; moreover, the chemical constituents of *N. incerta* in salt water were different from the same diatom when cultivated in fresh water.

Keywords: Diatom, *Navicula incerta*, Extract, Antibacterial, Different environment, GC-MS.

تقييم فعالية الدايتوم *Navicula incerta* المزروع في بيئات مختلفة ضد بعض الأنواع من البكتريا الممرضة

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الخلاصة

تعد الدايتومات مصدراً جديداً وقيماً للمركبات النشطة بيولوجياً ومنها المركبات التي تمتلك خصائص مضادة للميكروبات. لذلك ، أجريت هذه الدراسة لتقييم فعالية مستخلص الدايتوم *Navicula incerta* المضاد للبكتريا. عزلت الدياتوم من المياه المالحة في بحيرة ساوة الواقعة في جنوب العراق ، و زرعت في المياه المالحة ثم تم تكييفها وزراعتها في بيئة المياه العذبة ؛ حيث استخلصت الكتلة الحيوية المحصودة والمجففة ، وقد قيم النشاط المضاد للبكتيريا لكل مستخلص ضد عدة أنواع من البكتيريا المسببة للأمراض. تم تحليل المكونات الكيميائية للمستخلصات باستخدام تقنية الاستشراب الغازي – مطياف الكتلة. بشكل عام أظهرت النتائج أن مستخلصات المياه العذبة من دايتوم *N. incerta* لها فعالية مضادة للبكتريا أعلى من مستخلصه عند زرعه في الماء المالح. بالإضافه الى ذلك ، ثبط مستخلص الدياتوم النمو لسلالة المكورات العنقودية الذهبية التي كانت مقاومة لخمس مضادات حيوية مختلفة ؛ بالإضافه الى ذلك ، كانت المكونات الكيميائية لـ *N. incerta* في المياه المالحة لنفس الدياتوم مختلفة عند زراعتها في المياه العذبة.

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Introduction

The purpose of using algae for therapeutics has a long history, dating back in China in 2700 B.C. These algae were used to treat numerous diseases since it contains fatty acids, vitamins and biologically active compounds [1].

Many studies had led to the isolation and subsequent identification of active compounds. Alterations in antimicrobial activity of algal extracts may reflect different seasons, habitats, reproductive stage and utilized solvent of the extracts [2].

The Bacillariophyta (Diatom) is considered one of the main algal group on which so many studies had focused due to its availability [3], and production of various biologically active compounds that can be nominated for antibiotics production [4]. Diatoms are considered to be a good source of many yet underexplored antimicrobial substances; Furthermore, developments in algal culture techniques over the last decades have made diatoms a unique candidate over many other marine organisms, as diatoms can be cultivated under circumstances that maximize the production of the desired compound [5]. A global epidemic has arisen from the resistance of pathogenic bacteria for many of the known antibiotics. Marine algae by-products have shown promise as candidates in the novel, antibacterial drug discovery. The effectiveness of such compounds, their mechanism of action, applications as antibiotics, disinfectants, and inhibitors of foodborne pathogenic and spoilage bacteria [6]

This study aimed to investigate the antibacterial activity of *Navicula incerta* cultivated in different environments.

Materials and methods:

Sample collection

Water samples were collected from the brackish water of Sawa Lake, which is located in southern of Iraq (Al-Muthanna province), The collected samples were identified using a compound microscope and depending on algae keys [7, 8].

Biomass production

Navicula incerta was grown in artificial salt water (F/2 medium) prepared according to Guillard [9]. One liter of the diatom culture was transferred to 40 L F/2 media containing glass pools; the pools were incubated under illumination for 2 weeks. The diatom was then transferred to artificial fresh water (modified CHU-10 medium) prepared according to Kassim *et al*. [10] and the process was repeated for three successive generations in order to adapt the diatom for fresh water. After that; 2 liters of the adapted diatom was transferred to 80 L modified CHU-10 media containing glass pools; the pools were incubated under illumination for 2 weeks.

Extraction

The cultivated diatom from both environments was harvested and subsequently dried; after that, the dried biomass was extracted in Soxhlet apparatus using a mixture of ethanol and acetone (1:1) as a solvent and according to Chu *et al* [11].

Investigating the effect of crude diatom extract using agar dilution method

Agar dilution method was adopted to investigate the antibacterial activity against several species of pathogenic bacteria (*pseudomonas aeruginosa*, *Enterobacter cloacae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans*, *Acinetobacter lwoffii*, *Sphingomonas paucimobilis* and *Shewanella algae*) as follows: Different concentrations of diatom extracts ($0\text{-}50\times10^4$ µg/ml) were prepared in Muller Hinton agar plates. A volume of 2µl of an overnight bacterial culture with turbidity equals to McFarland standard no. 0.5 was spotted onto the agar surface and incubated at 37ºC for 24h. after that the minimum inhibitory concentration (MIC) was recorded at the lowest concentration with no visible growth. The antibiotic sensitivity of the bacterial isolates against (Gentamicin, Cefotaxime, Imipenem, Ceftriaxone, Ciprofloxacin and Nalidixic acid) was also tested using kirby bauer method [12]. chemical constituents of each extract were analyzed using Gas chromatography–mass spectrometry technique (GC-MS).

Results and discussion:

The result revealed that the extract of *Navicula incerta* cultivated in salt water contains several compounds with antibacterial activity (Appendix 1); Acetonyldimethyl-carbinol among other compounds extracted from Artemisia lavandulaefolia have inhibitory effects against several species of pathogenic bacteria [13]. Moreover, Keiko *et al*. [14], have mentioned that the growth of *E. coli* and *Staphylococcus aureus* was inhibited using Diethyl acetal compound.

The results obtained from GC/MS analysis of *Navicula incerta* extract cultivated in Fresh water (Appendix 2) revealed several compounds with potential antibacterial activity; Diethyl acetal among other compounds revealed antibacterial activity [15].

According to Zhang *et al*. [13], Acetonyldimethylcarbinol has demonstrated antibacterial activity by inhibiting the growth of *Bacillus subtilus*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus aureus*. According to Lamba [16], ketonic compounds inhibited the growth of *Salmonella typhimurium* and thus it could have contributed to the antibacterial activity of *Navicula incerta* salt water extract. Moreover, Nweke *et al* [17] and Alva-Murillo *et al* [18] mentioned that Tridecyne and Hexanoic acid respectively have antibacterial potential.

It can be concluded that the chemical constituents of the same diatom extracts that were cultivated in different aquatic environment differ greatly, and such difference may be attributed to the difference in the nutrients that were supplied in both fresh and saltwater environment, this variation is responsible for altering metabolic pathways in the diatom to produce different compounds.

Cultivation condition of microalgae affects both the biomass and lipid productivity along with cost-effectiveness and the composition of nutrient media (Nitrogen, carbon, phosphorus and trace metals) was one of the most significant factors that affect growth parameter and biochemical composition of microalgae [19]. A study by Chia *et al* [20] stated that the biochemical composition of algae is greatly influenced by the differences of the supplied media.

Salinity was another important factor that alters the biochemical composition of algal cells (salinity refers primarily to sodium chloride concentration unless otherwise specified). Exposing diatoms to lower or higher salinity levels than their natural (or adapted) levels can change growth rate and alter the composition. For example, higher salinity increases the algae lipid content [21].

Investigating the effect of crude diatom extract

The result revealed different levels of susceptibilities to the different concentrations of extracts (Table-1).

Table 1- Minimal inhibitory concentration (µg/ml) of diatom extracts.

Id	Bacterial species	*N. incerta* (salt water)	*N. incerta* (fresh water)	*P-value*
1.	*Staphylococcus aureus*	50000	30000	0.0001 **
2.	*Staphylococcus epidermidis*	40000	30000	0.0001 **
3.	*E. coli*	40000	30000	0.0001 **
4.	*Streptococcus mutans*	30000	30000	0.0001 **
5.	*Pseudomonas aeruginosa*	20000	30000	0.0001 **
6.	*Acinetobacter lwoffii*	10000	30000	0.0001 **
7.	*Sphingomonas paucimobilis*	20000	30000	0.0001 **
8.	*Shewanella algae*	40000	30000	0.0001 **
P-value		0.0001**	NS	---

** (P<0.01).

Statistical analysis revealed that significant differences (P<0.01) were observed between the activity of each diatom extract against all of the tested bacterial species. It was noticed that significant differences were also recorded between the susceptibility of each bacteria to the extract of *N. incerta* in salt water; nevertheless, no significant differences (P>0.01) were observed between their susceptibility to extract of *N. incerta* in fresh water.

The results revealed that the extract of *Navicula incerta* cultivated in salt water posed the greatest activity against *Acinetobacter lwoffii* with MIC of 10000 µg/ml, while the recorded MIC against *Pseudomonas aeruginosa* and *Sphingomonas paucimobilis* was 20000 µg/ml, on the other hand; The MIC values against *Streptococcus mutans* and each of *Staphylococcus epidermidis*, *Enterobacter cloacae* and *Shewanella algae* were 30000 and 40000 µg/ml respectively. However; the extract revealed a modest activity against *Staphylococcus aureus* with MIC value of 50000 µg/ml (Figure-1)

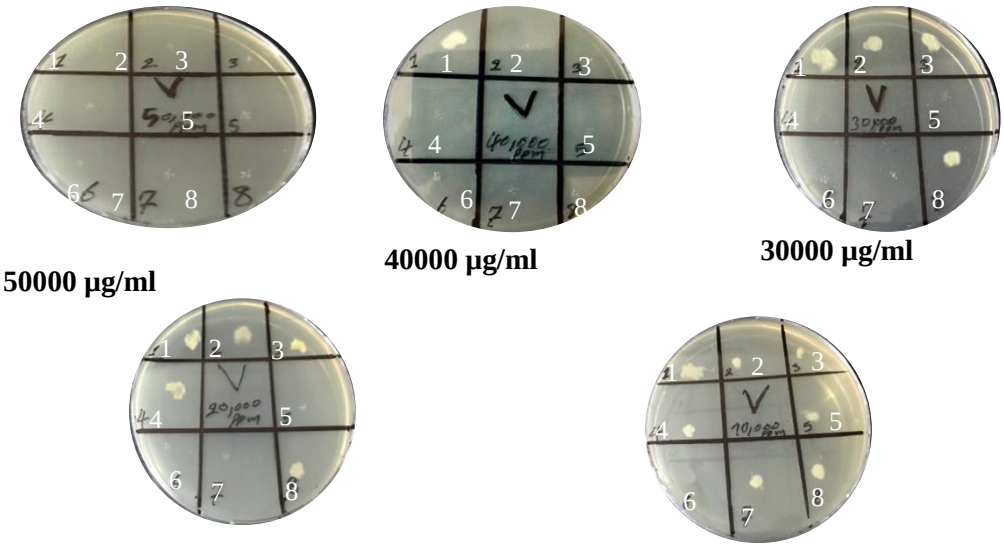

Figure 1-MIC of *Navicula incerta* salt water extract. Where 1. *S. aures* 2. *S. epidermidis* 3. *E. cloacae* 4. *S. mutans* 5. *P. aeruginosa* 6. *A. lwoffii* 7. *S. paucimobilis* 8. *S. algae*

It was found that the extract of *Navicula incerta* in fresh water was highly active against all the bacterial species that were included in this study at a fixed MIC value of 30000 µg/ml (Figure-2)

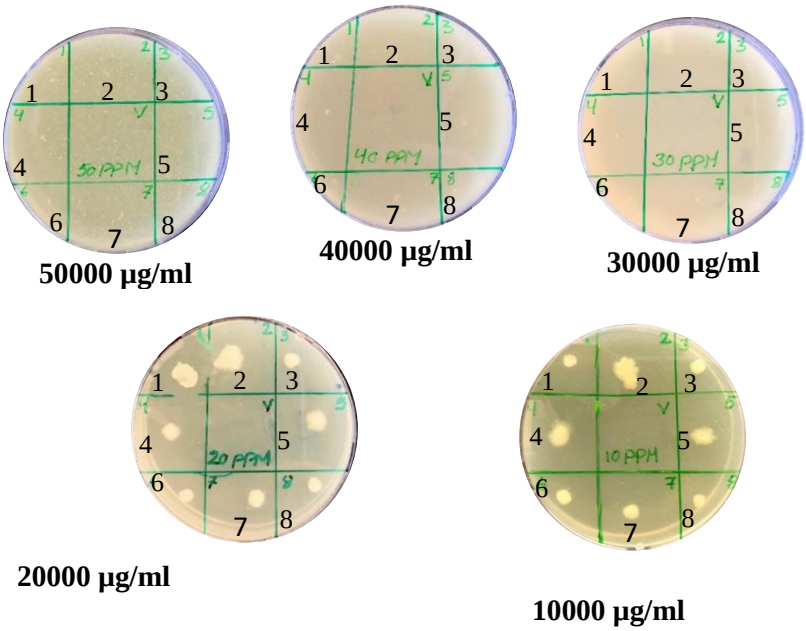

Figure 2- MIC of *Navicula incerta* fresh water extract. Where 1. *S. aures* 2. *S. epidermidis* 3. *E. cloacae* 4. *S. mutans* 5. *P. aeruginosa* 6. *A. lwoffii* 7. *S. paucimobilis* 8. *S. algae*

A study by Apsari and Paramita [22] on the extract of *Navicula sp*. collected from Bali Strait near Indonesia revealed that against *S. aureus* diatom extracts have high antibacterial activity with MIC of 2500 µg/ml thus suggested that it can be utilized as sources for the discovery of new antibiotics.
Okunowo *et al* [23] mentioned that the Methanolic extract of *Navicula* sp. has high antibacterial potential as it inhibited the growth of *Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus cereus*, *Klebsiella pneumoniae*, *Salmonella typhi* and *Enterobacter cloacae* with MIC values between < 2000-10000 µg/ml.

Findlay and Patil [3] stated that a novel ester compound isolated from *Navicula delognei* show significant antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella typhimurium*, and *Proteus vulgaris*.

Generally, it can be concluded from the findings of this study that the fresh water diatom extract was more efficient than the extract of salt water diatom, this could be due to the differences in the chemical makeup of these diatoms in fresh water environment according to GC/MS analysis.

Also, Microalgae biomass and chemical composition can vary due to the environmental circumstances, variation in salinity levels between fresh and salt water environment could result in subsequent variation in the chemical substances produced by diatoms; those of which could be in charge of the antimicrobial activity [24].

Antibiotic susceptibility testing

The results indicated that *S. aureus* has the highest resistance percentage while *S. epidermidis* has no resistance (Table-2).

Table 2-Bacterial Susceptibility to Antibiotics

Antibiotic / Isolate	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Enterobacter cloacae*	*Streptococcus mutans*
Gentamicin	R	I	R	R
Cefotaxime	R	S	S	S
Imipenem	S	S	I	S
Ceftriaxone	R	S	R	R
Ciprofloxacin	R	S	S	S
Nalidixic acid	R	S	I	R
Antibiotic / Isolate	*Pseudomonas aeruginosa*	*Acinetobacter lwoffii*	*Sphingomonas paucimobilis*	*Shewanella algae*
Gentamicin	R	R	S	R
Cefotaxime	R	S	R	I
Imipenem	S	S	I	I
Ceftriaxone	R	R	I	R
Ciprofloxacin	I	S	S	S
Nalidixic acid	S	I	R	I

R: Resistant; I: Intermediate; S: susceptible

Although *S. aureus* isolate was resistant to five different antibiotics, it was inhibited by the diatoms extract with different concentrations regarding the environment of the Diatoms. Moreover, both of *Streptococcus mutans* and *Pseudomonas aeruginosa* were resistant to three different antibiotics, they were also inhibited by the diatoms extract.

The discovery of novel antibacterial compounds that can be incorporated into new medicines is one solution to the global problem posed by antibiotic-resistant bacteria, such as multidrug-resistant *Staphylococcus aureus* (MRSA) [25]. In this context, microalgae constitute a potential source of novel antibacterial compounds [26] and a good choice for combating antibiotic resistant bacteria and fungal infections areas [27].

Several species of microalgae have shown antibacterial activities, attributed to different chemical classes of metabolites such as indoles, terpenes, phenols, volatile halogenated hydrocarbons or long chains unsaturated fatty acids [28]. These substances of algal origin offer the possibility to synthesize new drugs that provide an alternative against resistant pathogens to antibiotics known to date [29].

Conclusion: Generally, it can be concluded that fresh water extract is more efficient than the salt water extract of *N. incerta*. Furthermore, different chemical profiles were obtained from the same diatom when cultivated in different environments; this emphasizes the clear impact of the environmental contents on the chemical constituents of the diatom. Moreover, the inhibition of *S. aureus* by *N. incerta* extracts compared with its resistance to five types of antibiotics indicated the possibility of using the diatoms extract in the antibiotic industry from natural sources and according to their contents of bioactive materials.

Appendices:

Appendix 1-GC-MS Analysis of *Navicula incerta* cultivated in salt water extract.

Id	Name	R.Time	I.Time	F.Time	Area	Area%	Height	Height%
1	6-Heptene-2,4-diol	2.097	2.033	2.117	435897	8.82	115036	4.51
2	Acetone	2.139	2.125	2.158	55486	1.12	59360	2.33
3	Acetone, ethyl methyl acetal	2.250	2.200	2.267	611128	12.36	456230	17.88
4	Hexanoic acid, ethyl ester	2.363	2.325	2.392	236189	4.78	140002	5.49
5	Acetic acid, 2-propenyl ester	2.592	2.558	2.642	47172	0.95	27532	1.08
6	2-Pentanone, 4-hydroxy-4-methyl	2.751	2.717	2.800	306425	6.20	120861	4.74
7	Azomethane	2.874	2.850	2.900	76233	1.54	55807	2.19
8	Isobutenyl methyl ketone	3.106	3.075	3.142	418362	8.46	236984	9.29
9	Tetrachloroethylene	3.313	3.275	3.342	955674	19.33	521016	20.42
10	Acetonyldimethyl-carbinol	3.693	3.667	3.742	111977	22.65	544223	21.33
11	Benzylamine, N-(3-chloro-2,2-dimethyl-1-phenylpropylidene)	4.175	4.150	4.225	26840	0.54	18517	0.73
12	Methoxy acetaldehyde diethyl acetal	5.199	5.167	5.225	22756	0.46	14255	0.56
13	2-Naphthalenol	14.512	14.417	14.633	290952	5.89	73024	2.86
14	Oxalic acid, butyl propyl ester	18.573	18.542	18.600	17929	0.36	10795	0.42
15	Isoamyl nitrite	19.389	19.358	19.425	25893	0.52	12184	0.48
16	Pentadecanoic acid, 2,6,10,14-tetramethyl-, methyl ester	19.701	19.658	19.733	52985	1.07	31045	1.22
17	3H-Pyrazol-3-one, 2,4-dihydro-2-phenyl-4-(phenylmethylene)	22.217	22.183	22.267	101180	2.05	47293	1.85
18	Heptane	24.783	24.750	24.817	37691	0.76	20873	0.82
19	Hexanal, 3-methyl-	26.346	26.308	26.383	36388	0.74	17339	0.68
20	Sulfurous acid, hexyl octyl ester	27.533	27.500	27.567	38161	0.77	19365	0.76
21	1-Iodononane	29.622	29.583	29.675	30725	0.62	9903	0.39

Appendix 2- GC-MS Analysis of *Navicula incerta* cultivated in fresh water extract.

Id	Name	R.Time	I.Time	F.Time	Area	Area%	Height	Height%
1	Diethyl acetal	2.850	2.808	2.892	19507450	68.4	12219288	80.67
2	Isobutenyl methyl ketone	3.106	3.075	3.142	419671	3.09	236984	9.29
3	Acetonyldimethylcarbinol	4.857	4.808	4.908	3102776	22.8	1489473	9.83
4	1-Dodecyne	26.084	26.050	26.117	215890	1.1	128039	0.85
5	1-Tridecyne	26.354	26.325	26.392	148391	2.16	81934	0.54
6	1-Pentadecyne	26.559	26.525	26.592	294628	0.87	167968	1.11
7	Hexanoic acid, ethyl ester	27.695	27.667	27.733	118603	1.58	63391	0.42

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