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The effect of microalgae extraction on bacterial species isolated from seminal fluid of sexually- active males in Baghdad

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Abstract

Infection of the uorgientailc tract for the healthy man can direct effect on the motivation of the semen fluid which consequence effect on the man fertility. Bacterial infection on the main cause of the urogenital infection, particularly gram positive and gram negative. Microalgae (Chroococcus and chlorella) are known to produce compounds with anti- microbial activity against several types of bacteria. The aim of this study was to evaluate the antimicrobial and ant biofilm activity of ethanol extracellular and intracellular extracts obtained from the microalgae against a bacterial species isolated from a healthy sexual man in Baghdad. In addition to effecting of organic extraction associated with the biofilm-related infections. The partially purified ethanol extract obtained from chlorlla and Chroococcus algae isolated from the seminal fluid of 40 healthy sexual patients suffering from urogenital infection around Baghdad hospital Iraq. 20 isolated bacterial species were retained which were *Staphylococcus aureus*, *Escherichia coli* ,*Pseudomomas aeroginosa, Klebsiella pneumonia, Staphylococcus aureus, Klebseilla* and *Pseudomonas*. Biofilms of all bacterial species were found to be susceptible to the algal ethanol extract.

Keywords: Microalgae extraction, Intracellular, Extracellular, Biofilm, Seminal fluid

Introduction

Microalgae showed distinguished forms, in fact, appears as a single cells, colonies or extended filaments. Microalgae represent an essential role in the food chain in aquatic ecosystems; they have the notable ability to take up H_2O and CO_2 that, with the sunlight energy to synthesize the organic material which then release as a metabolite (Kaur *et al.*, 2010).

Microalgae universally distribute thought out different atmosphere, where can survive various physical and chemical environmental conditions (Kaur *et al.*, 2010), hence, they may grow essentially under all environmental conditions available, ranging from freshwater to extreme salinity, and can survive in the This requirement wide spectrum of the microalgae distribution makes the algae a unique requirement for the production of a novel cheap metabolite which involve in blue biotechnology (Josiane, 2009).

Microalgae has been used for disease curing for long time ago. Their therapeutic activity attributed to compound including indole, phenol, octagons (Butcher, 1959; Bhattacharyya and Kepnes, 2011). The universally growing of bacterial resistance eats a wide span anti-microbial agent has brought attention to a necessity to find a novel microbial agent (A-Mathkhury and Abed Assal, 2012; Saising *et al.*, 2012). However, a the essential factor for employing the microalgae product in the health sector relies on the key factor for their eventual economic feasibility which are able to produce biomass and metabolites to sufficiently high levels (Butcher, 1959; Passarelli and Sbalchiero, 2005). In most microalgae, the bioactive compounds are accumulated in the biomass (Intercellulr); however, in some cases, these metabolites are excreted into the medium; these are known as exo-metabolites (extracellular) (McWilliams *et al.*, 2012). Such compounds can present antifungal, antiviral, antialgal, antienzymatic, or antibiotic actions also having industrial, therapeutic and agricultural significance, Many of these compounds (cyanovirin, oleic acid, linolenic acid, palmitoleic acid, vitamin E, B12, β-carotene, hycocyanin, lutein and zeaxanthin) have antimicrobial antioxidant, and anti-inflammatory capacities, with the potential for the reduction and prevention of diseases (Butcher, 1959).

Micro algae produces the various antimicrobial active substances from the green alga, Chlorella vulgaris and the Cyanobacterium Pseudanabaena

sp. (Shimizu, 1993). Cyanobacteria from local habitats seem to be a source of potential new bioactive substances that could contribute to reduce of the number of bacteria، fungi, viruses and other microorganisms (Newman *et al.*, 2003).

Hence, attention has been paid to a more detailed understanding of the mechanisms underlying antimicrobial resistance – as well as to improved methods to detect resistance, new antimicrobial options for treatment of infections caused by resistant microorganisms, and methods to prevent the emergence and spreading of resistance in the first place. The key factor of a sustained increase of the bacterial infections has been associated with the biofilm mode of growth of pathogenic bacteria. In fact, current evaluations propose that the commonality of human infections includes biofuels (Srinivasakumar and Rajashekhar, 2009). Inside the biofilm bacteria supported by a substantial degree of guarded Within a biofilm, against challenging environmental conditions, natural and synthetic antimicrobials, chemical insults, mechanical removal, bacteriophages, external predation and elements of the body's immune system such as leukocytes (Newman *et al.*, 2003). In fact, biofilm associated infections frequently fail to respond to standard antimicrobial therapy depending on the research that comparing the effect of the same antibiotic on the bacterial in planktonic and biofilm form. As a consequence there has been a continuous decrease in the number of new antibacterial drugs approved for marketing globally with an 88% drop in the approval of novel systemic antibiotics since the mid-1980s (Srinivasakumar and Rajashekhar, 2009). This scenario points to the likelihood of a substantial increase in morbidity and mortality worldwide, justifying and necessitating renewed interest in research aimed at the discovery of novel anti-biofilm compounds and strategies focused on countering the emergence of antimicrobial resistance.

The aim of the present study was the assessment of antimicrobial activity of *Chlorella vulgaris* and *chlrococcus minor* of ethanol extract against bacteria isolate from the semen human fluid of human.

Materials and Methods

Sample collection and identification of bacterial strains: Twenty clinical bacterial strains were isolated from a sexual healthy patient suffering from Genitourinary tract infections in outward patients form hospitals around Baghdad, Iraq, isolates were (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeroginosa, Klebsiella pneumonia*). The Bacterial strains were streaked directly on Blood agar petri-dishes and incubated at 37^{O}C for 24hrs. Further identification tests were involved included morphological characteristics, biochemical tests and Api staph, Api 20E (Adejuwon *et al.*, 2009).

Minimal inhibition concentration and antibiotic sensitivity test: Different standard antibiotics were associated to test the susceptibility of each bacteria for the specified antibiotics such as (Bacitracin, Cefepime, Clindamycin, Cefepime, Ciprofloxacin, Gentamicin, Erythromycin, Methicillin, Levofloxacin, Doxycycline, Vancomycin, Erythromycin. An antibiotic sensitivity test was done using disc diffusion method, the diameter of zone of inhibition produced by each antibiotic disc was measured via a calliper and recorded. Bacterial isolates were categorized as resistant or sensitive based on CLSI (2007) and Adejuwon *et al.* (2009).

Minimal inhibition concentration (MIC): Was done according to Sendamangalam (2010).

Algae isolation and identification:

Prepare dried alga: *Chroococcus minor* and *Chlorella vulgaris* were isolated from AL-Mustansiriya University Gardens by Patterson Method (Eftekhar and Specrt, 2009). The algae identified by using an optical microscope (Olympus compound) according to Mulla and Reviwala (2011). Algae samples were cultured using ch-10 was used for algae growth (Table 1).

Table (1): The concentration of the compounds of modified chu 10 medium

concentration g/l	Salt	concentration g/l	salt
	B-micronutrient		A-macronutrient
1.00	EDTA . Na_2	5.8	Sodium Meta Silicate
2.86	H_3BO_3	57.56	$Ca(NO_3)_2.4H_2O$
1.81	$MnCl_2.4H_2O$	10	K_2HPO_4
0.222	$ZnSO_4.7H_2O$	25	$MgSO_4.7H_2O$
0.390	Na Mo $O_4.5H_2O$	4.36	EDTA .Na_2
0.079	$CuSO_4.5H_2O$	3.15	$FeCl_3.6H_2O$
0.0494	$Co(NO_3)_2.6H_2O$	20	Na_2CO_3

serial dilution and streaking for techniques CLSI (2007) and Eftekhar and Specrt (2009) were used for the isolation and purification of algae, which cultivation constant laboratory condition (temp 25°C, 200 Micro Einstein / m^2 intensity of illumination/ Sec for a period of 6 to 18hrs Lighting: darkness) (Saising *et al.*, 2012). The culture kept in the above condition for two weeks, all prepared algae culture centrifuged using an ordinary centrifuge 3000rpm for 15min. The sediment, dry at 40°C for 48hrs (Srinivasakumar and Rajashekhar, 2009).

Extraction of extracellular and intracellular active substance: 1gm of dried algae *C. minor* and *C. vulgaris* were added in 250ml of ethanol using 250ml conical flask, prepared solution shake for 2hrs (25°C, 70RPM) using shaking incubator. The supernatant centrifuge at 6000RPM for 15min, then sediment, dried using a rotary evaporator at a temp. of 40°C for 10hrs. Finally product be weighted and used in all experiments. All experiment has done in triplicate.

Biofilm formation assay: All isolates were subjected to biofilm production.

1- Congo red agar method: Qualitative evaluation of biofilm producers using the Congo red agar method to detect slime production was performed as follows: The medium is comprised of brain heart infusion broth (Hi media /India) 37g/l, sucrose (BDH / England) 50 g/l, congo red (Fluka) 0.8g/L and agar (Biolife /Italy) 10g/L. Inoculated plates were incubated at 37°C for 24hrs. Slime producing strains presented black colonies while non-producing strains developed red colonies (Saising *et al.*, 2012).

2- Microtitre plate method: Studied *Staphylococcus aureus* strains cultured in Brain Heart Infusion (BHI) broth (Hi media /India) incubated at 37°C for 18hrs, after that bacterial culture was diluted in BHI broth and adjusted in comparison to MacFarland tube no. 0.5. 200Ml bacterial culture was used to inoculate pre-sterilized 96-well polystyrene Microtiter plates and incubated for 48hrs. at 37°C. After incubation, all wells were washed with sterile physiological saline to remove unattached cells. Afterward, 200µl of 1% crystal violet was added to each well at room temperature. Wells rinsed with 200µl sterile physiological saline. This process was replicated three times.

200µl of ethyl alcohol were involved to remove the excessive stain (crystal violet) bounded to the biofilm. The absorbance of microliter plate was determined at 540 nm using an ELISA reader (Human/German). Controls were performed with crystal violet binding to the wells exposed only to the culture medium without bacteria. All the assays were performed in triplicates (Holt *et al.*, 1994; McWilliams *et al.*, 2012).

The cut-off optical density (ODc) for the microtitre-plate is defined as three standard deviations above the mean OD of the negative control. Strains were classified as follows: OD ≤ ODc non-adherent, ODc < OD ≤ 2 x ODc weakly adherent, 2 x ODc < OD ≤ 4 x ODc moderately adherent, 4 x ODc < OD strongly adherent).

In vitro inhibitory effect of extracellular and intracellular algae extraction on different bacterial species biofilm: Biofilm inhibition carried out in 96 wall plates adopting the modified method of biofilm inhibition spectrophotometric assay (Newman *et al.*, 2003) (9):All isolates cultured in Brain Heart Infusion (BHI) broth (Hi media /India) incubated at 37°C for 18hrs, after that bacterial culture was diluted in BHI broth and adjusted in comparison to MacFarland tube no. 0.5. Two hundred microliters of this bacterial culture were used to inoculate pre-sterilized 96-well polystyrene microtiter plates and later incubated for 48hrs at 37°C. After incubation, all wells were washed with sterile physiological saline for the elimination of unattached cells. Then, before the staining step, the extracelluer and entracelluer extraction of algae was added to biofilm containing wells: Subsequently, the tray was incubated for another 24hrs after incubation period all wells were washed and stained as procedure described above (Maldonado *et al.*, 2007; Dheepa *et al.*, 2011).

Results and Discussion

Bacterial isolates: A total of 40 seminal fluid was collected from male patients with urogenital infection. The age of patients ranged from 15 to 35 years, mean age (25.1 years); 20 samples were positive for microorganisms, whereas 20 were negative. A total of 20microorganisms was found, including 15 Gram-negative and 5 Gram-positive bacterial isolates (Figure 1). summarizes list and distribution of pathogens isolated from the positive semen fluid. *K. peumoniae* had highest number of isolates (7), followed by *S. aureus* (5), *P. eruogenosia* (4), and *E. coli* (4).

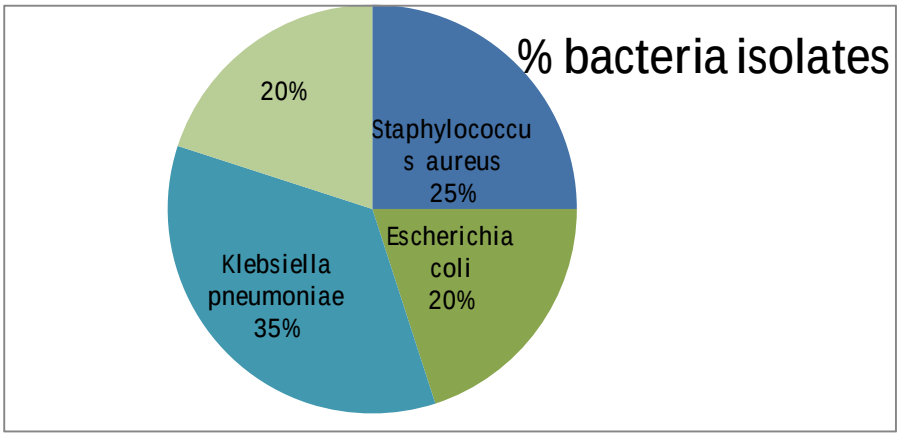

Figure (1): Percentage bacterial isolate

Susceptibility of bacterial isolates to antibiotics: Table (1) shows the patterns of susceptibility to antibiotics of main pathogens. *E. coli* and *S. aureus*. *E. coli* bacteria exhibited resistance to bacitracin, Nalidixic acid, Imipenem, Ciprofloxacin, Cefoxitin, Ceftriaxon, Cefotaxime and *S. aureus* showed 60% resistance to cefiepim, Ceftazidime and Erythromycin, while *E. coli* bacteria showed a 100% resistance for above antibiotics, while *K. pneumonia* and *sp. eurogeinosa* was 100% resistance to Cefotaxime, Cefoxitin, Ceftriaxon. Imipenem and ciprofloxacin were the most active antibiotics against *Ps. aeruginosa* (100% and 85% sensitive strains), followed by Rifampin (85%), Trimethoprim (14%) (Tables 2 and 3).

Biofilm assay: 20 isolates were biofilm producers (Table 3). One out of 5 strains of *S. aureus* were shown to be able to induce strong biofilm production and two isolates showed an ability to induce a moderate biofilm (slight producers). While two *E. coli* bacteria produce a strong biofilm. Only one isolate has a slightly producer for biofilm. *K. peumoniea* were the higher producer for biofilm with 6 isolated yield a strong producer for biofilm (Table 4).

Antibacterial activity of Chroococcus and Chloroella extracts: Table (4) shows optical density values obtained after treatment of main bacterial isolate form urogetical patient seminal fluid (*S. aureus*, *P. aeruginosa*, *E. coli* and *Klebsiella* sp.). OD values obtained after treatment of *S. aureus* strains with extracelluer extract of Chroococcus was (0.727- 0.19), while for the entracellular extract (0.727- 0.194).

Table (2): Percentage of antibiotic sensitivity test for *S.aureus* and *E.coli* bacteia

Antibiotic disc		*S. aureus* (5)		*E. coli (4)*	
		R%	S%	R%	S%
Amoxicillin-clavulanic acid	AMC	60	40	100	0
Bacitracin	B	100	0	-	-
Cefepime	FEP	60	40	100	-
Cefotaxime	CTX	100	0	100	0
Ceftazidime	CAZ	60	40	100	0
Cefoxitin	FOX	100	0	100	0
Ceftriaxone	CRO	100	0	100	0
Ciprofloxacin	CIP	0	100	75	25
Erythromycin	E	60	40	-	-
Imipenem	IPM	0	100	0	100
Methicillin	ME	100	0	_	_
Nalidixic acid	NA	_	_	100	0
Nitrofurantion	F	100	0	25	75
Rifampin	RA	100	0	0	100
Trimethoprim	TMP	40	60	50	50

Table (3): Percentage of antibiotic sensitivity test for *K. pneumoniae* and *Ps. aerogenosa* bacteia

Antibiotic disc		*K. pneumoniae* (7)		*Ps. aerogenosa (4)*	
		R%	S%	R%	S%
Amoxicillin-clavulanic acid	AMC	100	0	100	0
Cefepime	FEP	100	0	100	0
Cefotaxime	CTX	100	0	100	0
Ceftazidime	CAZ	100	0	100	0
Cefoxit	FOX	100	0	100	0
Ceftriaxone	CRO	100	0	100	0
Ciprofloxacin	CIP	14.28	85.71	50	50
Imipenem	IPM	0	100	0	100
Nalidixic acid	NA	100	0	100	0
Nitrofurantion	F	42.85	57.14	75	25
Rifampin	RA	14.28	85.71	50	50
Trimethoprim	TMP	85.71	14.28	75	25

A. B.

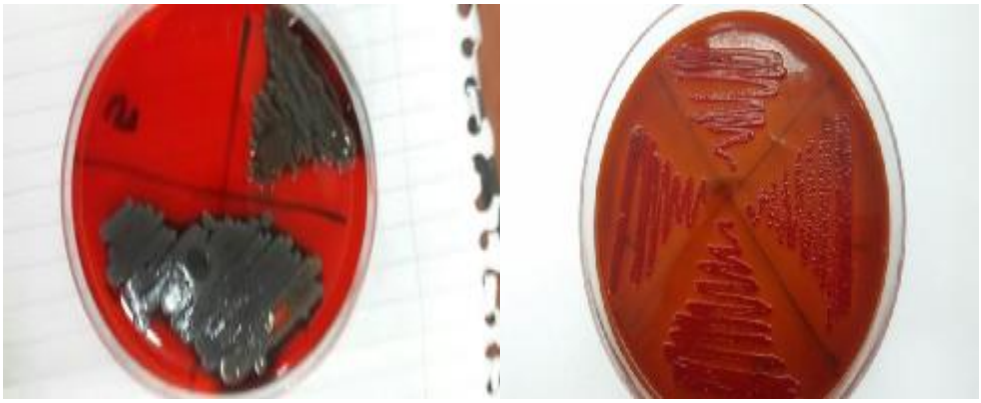

Figure (2): bacterial strain produce a biofilm on the MacConkey agar A. *K. peumoniea* B. *S. aureus*

Table (4): Optical density of biofilm bacterial isolates (490nm using the Eliza reader)

Bacteria Strains	O.D. (490nm)	Bacterial strains	O.D. (490 nm)
S. aureus 1	0.335^{m}	*K. peumoniae 2*	0.154^{n}
S. aureus 2	0.197^{n}	*K. pneumoniae 3*	0.128^{n}
S. aureus 3	0.727^{s}	*K. pneumoniae 4*	0.533^{s}
S.aureus 4	0.137^{n}	*K pneumoniae 5*	$o.346^{m}$
S.aureus 5	0.498^{m}	*K. pneumoniae 6*	0.845^{s}
E. coli 1	0.583^{s}	*K.pneumoniae7*	0.111^{n}
E. coli 2	0.595^{s}	*Ps. aeroginosa 1*	0.758^{s}
E. coli 3	0.153^{n}	*Ps. aeroginosa 2*	0.163^{n}
E. coli 4	0.171^{n}	*Ps. aeroginosa3*	0.675^{s}
K. pneumoniae1	0.468^{m}	*Ps aeroginosa 4*	0.101^{n}

For the *E. coli* 0.595^{s}-0.138 and 0.595^{s}-0.167 for extracelluer and entracelluer extract of *Chroococcu* and 0.595-0.138 for the extrcelluer and 0.595-0.167 for the intracellular extract of Chloroella respectivly. *Klebsiella pneumonia* 4 shows the optical density values obtained after treatment with Chroococcus extracellular 0.583- 0.098 and 0.583 – 0.122 for the intracellular extract. While for the Chloroella optical density values obtained after treatment with extracellular 0.583- 0.103 and 0.583 – 0.136 for the entracellular extract. However OD. values obtained after treatment of *Ps. aeroginosa* with Chroococcus extracellular 0.675- 0.099 and o.675 – 0.133 for the entracellular extract. While for *Chloroella* shows a significant decrease in eh OD values after treatment with extracellular 0.675- 0.123 and o.583 – 0.175 for the entracelluer extract.

Table(5): Optical density reading for bacterial strains after treatment with algae extract

No. Isolates	Before treatment	After Treatment			
		Chroococcus		*Chloroella*	
		Extra	Intra	Extra	Intra
S. aureus 1	0.335^{m}	0.044	0.107	0.133	0.164
S. aureus 3	0.727^{s}	0.129	0.194	0.199	0.209
S. aureus 5	0.498^{m}	0.095	0.199	0.102	0.198
E. coli 1	0.583^{s}	0.087	0.144	0.198	0.201
E. coli 2	0.595^{s}	0.138	0.176	0.166	0.197
K. pneumoniae 1	0.498^{m}	0.071	0.108	0.143	0.184
K. pneumoniae 4	0.583^{s}	0.098	0.122	0.103	0.136
K. pneumoniae 5	0.346^{m}	0.076	0.106	0.116	0.153
K. pneumoniae 6	0.845^{s}	0.134	0.153	0.188	0.193
Ps. aeroginosa 1	0.758^{s}	0.112	0.159	0.144	0.191
Ps. aeroginosa 3	0.675^{s}	0.099	0.133	0.123	0.157

In this study, *S. aureus* and *Sp. Aeruginosa* were the main agents of biofilm. *P. aeruginosa* was the main pathogen infection in mean notably. It can be supposed that the infection could be facilitated by hot and damp climate as well as by stanation of water into the EAC due to bathing.

To date, bacterial infections in the clinical practice are relevant to the biofilm formation by bacteria, and more than 60% of infections seem to be occurring in the presence of biofilms (Sendamangalam and Antibiofouling, 2010). Recent studies showed that urogenital infection is biofilm related (Tabak *et al.*, 2007). In our study, 25%, 20% and 35% of *S. areus, Sp euroginosa and K. pneumonia* were biofilm producers. Among Gram-negative bacteria, 25.8% of *Sp. aeruginosa* were seen to be producers or slight producers of biofilm.

The effect of algae extraction back to many reason such as:

A. phytochemical screening of the Chlorella vulgaris fraction are performed. Studyes reveals the level of Flavonoids, Tannin, Phenolic compounds (Gianluca *et al.,* 2015).
B. Chlorella vulgaris had the greatest frequency among the species that showed antibacterial and antifungal activity and exhibited the most prominent effect (Shabudeen *et al.,* 2015).
C. The antibacterial activity of the extracts could be due to the presence of different chemical agents that may include flavonoids and triterpenoids and other compounds of phenolic nature or free hydroxyl group, in addition to the presence of certain metabolites such as tannin, alkaloids protein, and flavonoids in the extract of cyanobacteria, also identified antimicrobial agents in alga Pithophora (Entesar, 2016).

In this study, the evaluation of the antimicrobial activity of extracts from *chlorocccus* against *S. aureus, Sp. euroginosa and K. pneumonia* and *E. coli* bacteria biofilm iformateion has been attempted. Results show that extracts from *chloroccous* and Chlorlla algae sp. have antimicrobial activity, notably against *SP. Aeruginosa*, lower activity was observed against *S. aureus*. The extracts, notably those from, showed activity on other bacterial species as well.

Studies on extraction of antimicrobials from algae began in 1940s. Subsequently, stated that algae speice produces a broad spectrum of antibiotic substances depending on the site of collection. Recently, extracts of *chlorIlla* did not show activity against *E. coli*, *P. aeruginosa*, and *S. aureus* only, but has a significant action against *Bacillus subtilis*.

Our results show that extracts *chlorella and clorrococcus* can produce compounds provided with antimicrobial activity useful for the treatment of diseases due to bacterial agents. Significantly interesting compounds having great pharmacological characteristics have been discovered in marine organisms and the research in this field is in progress.

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