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ANTIMICROBIAL ACTIVITIES OF *SPIRULINA PLATENSIS* EXTRACTS AND NANOPARTICLES MATERIAL AGAINST SOME PATHOGENIC BACTERIAL ISOLATES

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ABSTRACT

The algal species *Spirulina platensis* have the potential to produce a large number of antimicrobial substances that considered as suitable bio-control agents for plant pathogenic bacteria. The present study aimed to synthesize nano- material of *S. platensis*, use anti-bacteria material of *S. platensis* using solvent extracts and tested them against pathogenic bacteria by Standard well diffusion method for antibacterial activity.

Mullor Hinton Agar (MHA) plates and pure cultures of bacterial pathogens were grown in Nutrient broth at 37^{O}C for 18-24 hours. In the present, the tested positive and negative gram bacteria were isolated from water Omar Bek drainage water-Damietta branch of Nile River

The data of sodium-citrate extraction showed maximum zone of inhibition against all the bacterial isolated while extraction of S. platensis showed minimum inhibition zone against bacterial pathogens in comparison to other solvent extracts. Nano material recorded the highest zone of inhibition against *Yersinia pestis* (45mm) in comparison with Na-citrate against *Staphylococcus aureus* (20mm) as an organic solvent extract. The research concluded and recommended *S. platensis* should be considered as an economic antibacterial agent than using medical antimicrobials against pathogenic bacteria.

1. INTRODUCTION

Spirulina, a blue green alga is now becoming a health food along worldwide. It is an edible, microscopic, multi-cellular, filamentous, alkalophilic, photo auto trophiccyano bacterium that belonging to micro algae of the class Cyanophyta. It consists of a larger cell size for ease of cultivation, for ease of harvest and an easily digestible cell wall. They are considered as rich source of protein, vitamins, and minerals than any

other single cell protein. Dominating the micro-flora of alkaline saline waters with pH of up to 11.0 and they can exist in various types of habitats, namely soils; marches, thermal springs uses, fresh water, seawater and brackish, domestic, industrial wastewaters **(Jensen and Knutsen, 1993 and Marcello Nicoletti, 2016).**

They have been a nature source of medicinal agents for thousands of years. Modern drugs have been isolated from natural sources based on their uses in traditional medicine. Recently many screening of cyanobacteria, antibiotics and other pharmacologically active compounds have received considerable attention **(Haidan *et al,* 2016)**.

Spirulina as many other cyanobacteria species have the potential to produce a large number of antimicrobial substances that they are considered as bio-control agents of plant pathogenic bacteria and fungi. Algal organisms are rich source of structurally novel and biologically active metabolites that primary or secondary metabolites produced by these organisms may be potential bioactive compounds of pharmaceutical industry **(Yuliani *et al.,* 2021)**.

S. platensis produce a diverse range of bioactive molecules, making them a rich source of different types of medicines, **(Kapoor and Mehta 1993; Nasima*, et al.,* 2012).** The *Spirulina,* as a whole, has been known only for its nutritional value but their antimicrobial property of the C-phycocyanin has not been studied in detail in Indian context. Antimicrobial compounds found in cyanobacterial exudates include polyphenols, fatty acids, glycolipids, terpenoids, alkaloids and a variety of yet to be described bacteriocins **(Sherif *et al.,* 2021)**.

Secondary metabolites from cyanobacteria are associated with toxic, hormonal, anti-neoplastic and antimicrobial effects. The antimicrobial substances involved may target various kinds of micro-organisms, prokaryotes as well as eukaryotes. The properties of secondary metabolites in nature are not completely understood. Secondary metabolites influence other organisms in the vicinity and are thought to be of phylo-genetic importance. The methods that commonly applied for extraction are based on the agar diffusion principle using pour-plate or spread plate (Seeded plates) techniques. Antimicrobial effects are shown as visible zones of growth inhibition (Inhibition halos) **(McGill and Hardy, 1992).** The research aimed to synthesize nano-material of *S. platensis,* use anti-bacteria material of *S. platensis* algae using solvent extracts and tested them against pathogenic bacteria by Standard well diffusion method for antibacterial activity**.**

2. MATERIALS AND METHODS

2.1 Study area and Sample Collection

The study area includes about 119 km (73.94 miles) of the Damietta branch and extended from upstream of the Omar-Bek drain to Faraskour City as shown in **Fig.(1)**. It receives the outlet of three agricultural main drains (Omar Bek, Upper Serw and Drain No.1) where they uploaded with untreated sewage water from many villages. Omar Bek drain is about 130 km far away from Cairo. It serves about 43,000 feddan (one feddan=0.42 ha) of fertile lands and has a discharge rate of about 12,000 m /h. These wastewaters are collected from industrial, domestic, and agricultural effluents along this drain path from Zefta to Samanoud cities. Omar Bek was initially built as an agricultural drain by the 1980s, and its water quality was in normal ranges till the 1990s. After the 1990s, many environmental issues related to this drain started to be raised **(Ezzat and Elkorasey, 2020; Mostafa and Peters, 2016).**

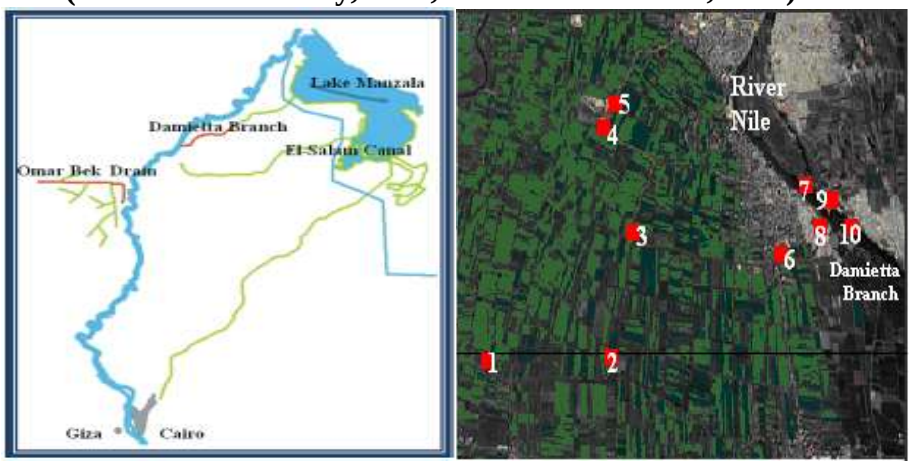

Fig.(1): Map of Study Area Location and Water Samples along Omar Bek Drain and Damietta Branch-Nile River

In this study, area under investigation ten water samples along Omar Bek drain and from Damietta branch of Nile River, during autumn (on 26 September 2016- from 8.00 a.m. to 10.00 a.m.- air temperature was 31^{o}C) as shown in Fig.(1). Sampling procedures were carried out according to Standard Methods for Examination of Water and Wastewater **(APHA, 2012)**. All collected samples were stored in an iced cooler box and delivered immediately to the Central Laboratory for Environmental Quality Monitoring, National Water Research Center "CLEQM-NWRC" where it has been analyzed.

2.2 Isolation, purification and characterization of bacteria isolates

2.2.1 Identification of Bacterial isolates:

For all wastewater samples, a volume of 100ml was filtered through 0.47µm pore sized filter using water pump. All selective media used were prepared according to **Bergeys Manual (1957)** in order to isolate and identify bacteria spp.

2.2.2. Morphological characteristics of Bacterial isolates:

Morphological characteristics of colonies color, Gram reaction. Cell Shape, Spore formation, and Motility and Diffusible pigment were investigated. Physiological and biochemical characteristics of pathogenic bacteria isolates were conducted according to tests in **Bergeys Manual (1957). Table (1)** illustrates the Morphological and biochemical characteristics of identified bacteria isolates from water of Omar bek drain.

2.2.3 Collection of Pathogenic Bacteria:

Eleven different bacterial cultures of Gram positive and Gram negative bacteria were isolated from water samples along Omar Bek drain that were:

Gram positive bacteria: *Staphylococcus aureus* and *Enterococcus faecalis.*

Gram negative bacteria: *Pseudomonas aeruginosa; Serratia liquefaciens; Enterobacter aerogenes; Klebsiella pneumonia; Shigella sonnei; Legionella pneumophila; Yersinia pestis; Moraxella catarrhalis and Hafnia alvei*

2.3 Determination of Antimicrobial Activity of *S. platensis*

2.3.1 Preparation of *S. platensis* extract:

Cells of *S. platensis* were cultivated in Zarrouk growth media **(Fig.2a) (Zarrouk, 1966)** at constant shaking at 30°C±2°C and pH 10 in light/dark conditions (16/8 hrs) with shaking of culture manually twice a day. Bacterial cells were harvested after 5-6 days and then washed twice in distilled water. The collected cells were preserved and the supernatant was discarded. The cells of *S. platensis* were stored at -20 until be used.

2.3.2 Phyto-chemical Extraction:

About 2 gm of algae fresh weight were added to 10 ml of the desired organic solvent (Na citrate; Ethanol; Hexane; DMSO and acetone) **(Fig. 2b, c)**, mixed well per each organic solvent and then the mixture was exposed to Sonication (Cycle 5 min on, 5min off, 1min on, power 100% on Ice). After Sonication the volume was completed into 100ml with worm water were add to the sonicated solution. Then, the solution was incubated for 16 hours at 30°C with shaking at 150 rpm. Water and methanol extraction were performed according to **(Tsibakhashvili, *et al.*, 2011).**

Table (1). Morphological and biochemical characteristics of identified bacteria isolates from water of Omar bek drain.

Test	L. pneumophila	Y. pestis	H. alvei	S. liquefaciens	P. aeruginosa	S. aureus	M. Catarrhalis	E. faecalis	E. aerogenes	K. pneumoniae	S. sonnei
Temp. Limits of growth	36°C	35-37°C	2.6°C-42°C	30-37°C	37°C, -/4°C and +/-41°C	15 - 45°C	28-37°C	10-45°C	40°C	36°C	35-37°C
Shape of colony	Rods and filaments	Raised irrigular	Straight	Straight rods	straight rods	Raised, circular and entire	large and kidney	Small-raised and entire	Rod	Rod	Short rods
Texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Pigmentation	-	-	-	-	Blue-green exopigment	Golden yellow	-	-	Yellow	-	-
Motility	+	-	+	+	+	-	-	-	+	-	-
O2 requirements	Aerobic	facultatively anaerobic	facultatively anaerobic	Aerobic	Facultatively anaerobic	Facultatively anaerobic	Aerobic	Facultatively anaerobic	Aerobic/ anerobic	Facultatively anaerobic	Facultatively anaerobic
Gram reaction	-	-	-	-	-	+	-	+	-	-	-
Cell Shape	Rods	Rods	Rod	Rods	Straight rods	Cocci in clusters and pairs	Kidney	Cocci in pairs short chains	Rod	Rods	Rod
Sporulation	-	-	-	-	-	-	-	-	-	-	-
Capsule	-	+	-	-	-	-	-	-	+	+	-
Catalase	+	+	+	+	+	+	+	-	+	+	+
Coagulase	-	+	+	+	-	+	NA	-	+	+	
Oxidase	+	-	-	-	+	-	+	-	-	-	-
Urease	-	-	-	+	-	+	-	-	-	+	-
Gelatin liquefaction	-	-	-	+	+	+	+	+	-	-	-
Starch hydrolysis	+	+	-	+	-	-	+	-	-	-	-
Phyenl amine deaminase	+	-	-	-	-	-	+	-	-	-	-
H_2S production	-	-	-	-	-	-	+	-	-	-	-
Heamolysis blood agar	β-Heamolysis	-	β-Heamolysis	β-Heamolysis	β-Heamolysis	β-Heamolysis	-	β-Heamolysis	β-Heamolysis	-	-
Nitrate reduction	-	+	+	+	-	+	+	-	+	+	+
Indol formation	-	-	-	-	-	-	-	-	-	-	-
Methyl red	-	+	+	-	-	+	+	-	-	-	+
Tween 80 hydrolysis	+	+	-	-	-	+	-	-	-	-	-
Voges-Proskauer	+	-	+	+	-	+	-	+	+	+	-
Citrate utilization	+	-	-	+	+	+	-	-	+	+	-
D-glucose	+	+	+	A/G	-/-	A/-	-	A/-	A/G	A/G	+
Sucrose	+	-	-	+	-/-	A/-	-	A/-	+	+	-
Mannose	+	+	+	+	-/-	A/-	-	A/-	+	+	+
Lactose	+	-	-	-	-/-	A/-	-	A/-	+	+	-
Mannitol	+	+	+	+	A/-	A/-	-	A/-	+	+	-

(+), positive result; (-), negative result; (A), (NA), Not Applicable; (A), acidproduction and (G), gas production.

2.3.4 Antimicrobial activity of the obtained algae nano silver

In vitro antimicrobial activities had been examined for 11 pathogenic bacteria using the agar disk diffusion method according to **(Attaie, *et al.*, 1987).** Inhibition zones of growth around the disks were measured after 24 hours of incubation at 37°C. The nano-particle activity was compared with some other of generic antibiotics.

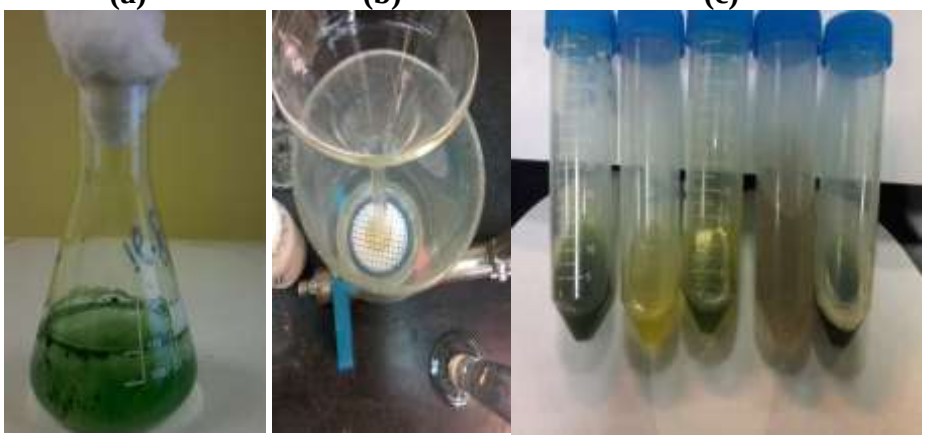

Figure (2): (a) *S. platensis* cultivation; **(b)** Extracting antibiotics from *S. platensis* cultivation; **(c)** antibacterial agents test of *S. platensis* against pathogenic bacteria.

2.3.5 Determination of Antibacterial Activity by Well Diffusion Method

The wet biomass of *S. platensis* (1g) was re-suspended in 500 ml Erlenmeyer flask with 100ml of 10^{-3} M aqueous silver nitrate (preparing 1mM of Silver nitrate ($AgNO_3$) solution: dissolving 0.169g of $AgNO_3$ (Sigma, 99%, 169.87g/mol) in 1 liter of de-ionized water.) by using de-ionized water (pH7) incubated at room temperature for different time intervals (1–5 days). **(Kalabegishvili *et al.*, 2012; Devina *et al.*, 2010).**

The AgNPs synthesized from *S. platensis* was tested for its antibacterial activity against pathogenic bacteria by standard well diffusion method in Mullor Hinton Agar (MHA) plates. Pure cultures of bacterial pathogens were grown in Nutrient broth at 37^{O}C for 18-24 hours.

Wells were done on Moller-Hinton agar plates by using gel puncture and plates were inoculated by scavenging bacterial pathogens in order to create a confluent turf of the bacterial growth. After incubation at 37^{O}C for 24 hours, diameter of zone of inhibition in millimeter around each well was measured **(Thomas *et al.*, 2012).**

In this study, we used *S. platensis* algae as natural antibacterial through phytochemical extraction with other organic solvents, against pathogenic bacteria. We used *S. platensis* as natural nano material through mixing algae with $AgNO_3$ solution, against pathogenic bacteria

3. RESULTS AND DISCUSSION

3.1 Antibacterial Activity of Organic Antibacterial Solvents of *S. platensis*

The antibacterial activity of *S. platensis* was determined against bacteria and the findings were furnished in the **Table (2), Figures (3 and 4).** The zone of inhibition of *S. platensis* extracts against bacteria was ranged between 5 mm to 45 mm at 200 µl. The Na-citrate extract of *S. platensis* showed the highest mean zone of inhibition (20 mm) against the Gram positive cocci *Staphylococcus aureus*, followed by *Enterococcus faecalis* (15 mm), in comparison to other organic solvents. The minimum zone of inhibition obtained from the acetone extract of *S. platensis* against bacterial pathogens was comparatively very less when compared to the other solvent extracts **(Saranraj, 2015).**

For Gram negative bacteria, the maximum zone of inhibition was recorded also in Na-citrate extract of *S. platensis* against *Yersinia pestis* (17mm) followed by *Serratia liquefaciens* (16mm), *Klebsiella pneumoniae* (15mm), *Legionella pneumophila* (13mm), *Moraxella catarrhalis* (11mm), both *Pseudomonas aeruginosa* and *Shigella sonnei* were recorded (10mm), *Hafnia alvei* (7mm) and *Enterobacter aerogenes* (5mm). No zone of inhibition was seen in DMSO and ethanol with both gram positive and negative bacteria, in agreement with data of **Saranraj, *et al.*, (2015). Kaushik and Chauhan (2008)** reported that extracts of *S. platensis* inhibited the growth diameter of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi and Klebsiella pneumoniae.* They used hexane, ethyl acetate, dichloromethane and methanol to obtain the phenolic extracts and the methanolic extracts had the best results. The methane extract of *Staphylococcus aureus* and *Escherichia coli* minimum inhibitory concentrations (MIC) were 128µg/ml and 256µg/ml respectively.

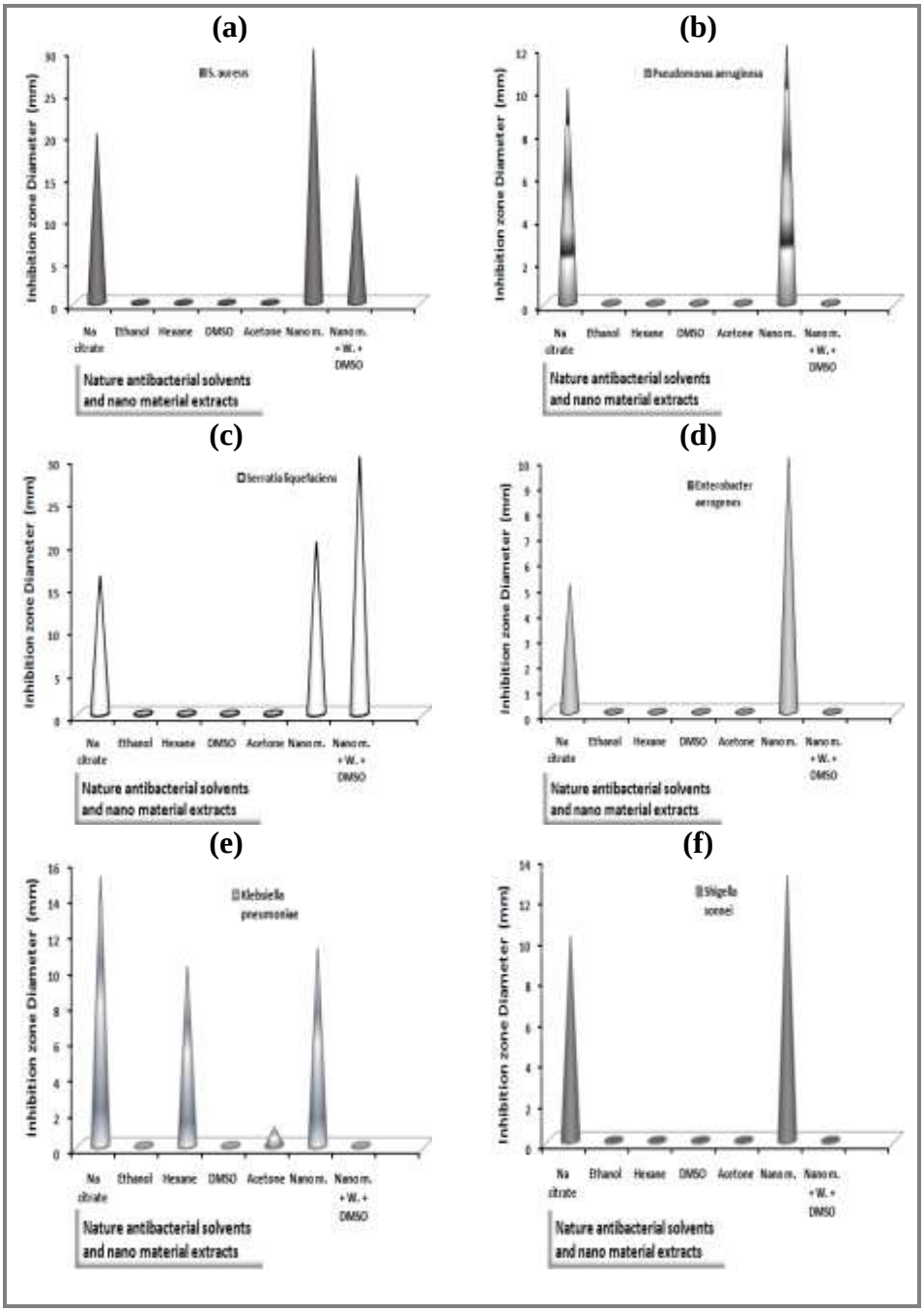

Figure (3) Resistance of **(a)** *Staphylococcus aureus*; **(b)** *Pseudomonas aeruginosa*; **(c)** *Serratia liquefaciens*; **(d)** *Enterobacter aerogenes* ; **(e)** *Klebsiella*

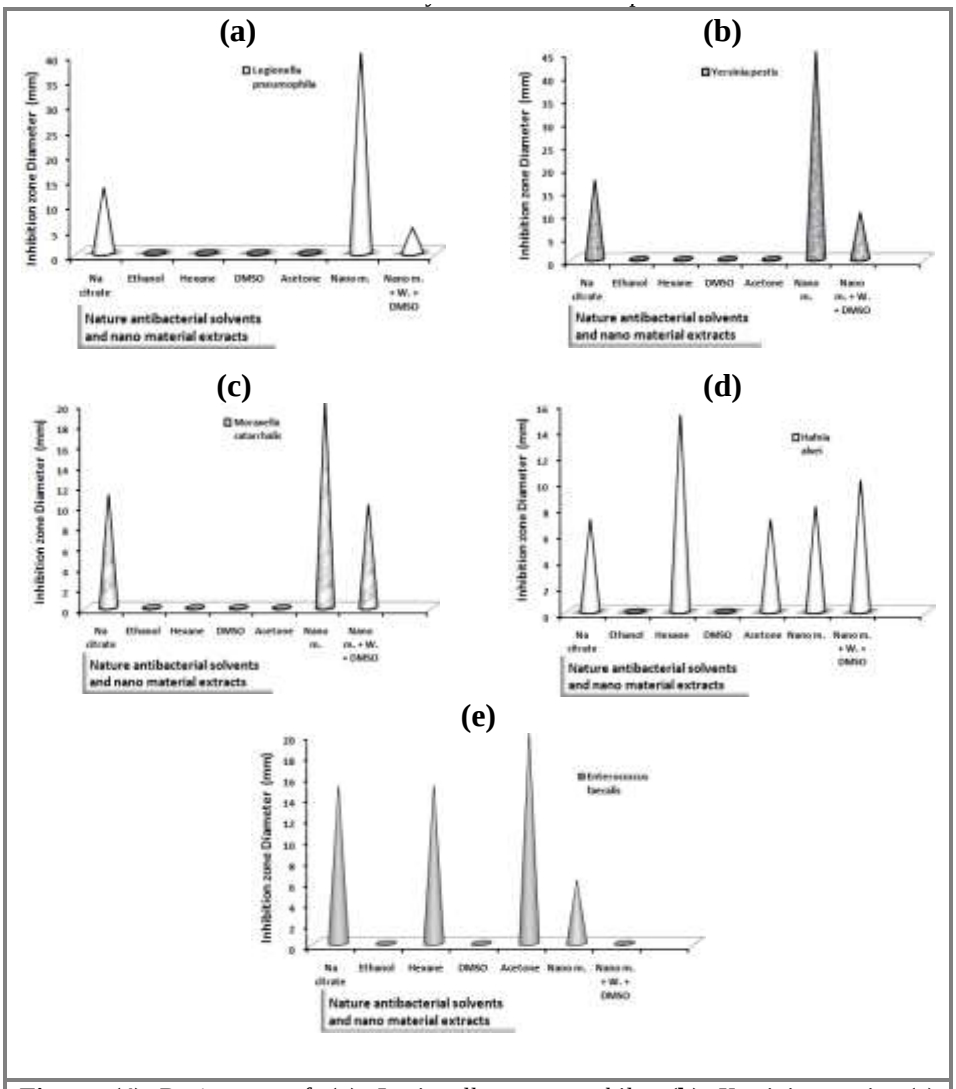

Figure (4) Resistance of **(a)** *Legionella pneumophila*; **(b)** *Yersinia pestis*; **(c)** *Moraxella catarrhalis*; **(d)** *Hafnia alvei* ; **(e)** *Klebsiella pneumonia*; **(f)** *Enterococcus faecalis* isolates against nature antibacterial solvents and nano material extract synthesized from *S. platensis* solution.

Parisi *et al.*, (2009) also found high antimicrobial activity of phenolic compounds extracted with methanol from *S. platensis* against Gram positive *Staphylococcus aureus*. **Vinay Kumar *et al.*, (2011)** examined the algal extracts in vitro for their antibacterial effects against (*Staphylococcus aureus* and *Salmonella typhimurium*) using Agar well

diffusion method and Paper disc diffusion method with concentration from 250ppm to 7000ppm and it noticed that all of these bacteria showed inhibition in the growth of these extracts.

Table (2): Antibacterial Activity of Organic Solvents and Nano Mmaterial Synthesized by *S. platensis*

Isolates	Organic Solvents + Algae					Algae as Nano Material	
	Na citrate	Ethanol	Hexane	DMSO	Acetone	Nano Material only	Nano Material + Water +DMSO
Staphylococcus aureus	20	-ve	-ve	-ve	-ve	30	15
Pseudomonas aeruginosa	10	-ve	-ve	-ve	-ve	12	-ve
Serratia liquefaciens	16	-ve	-ve	-ve	-ve	20	30
Enterobacter aerogenes	5	-ve	-ve	-ve	-ve	10	-ve
Klebsiella pneumoniae	15	-ve	10	-ve	10	11	-ve
Shigella sonnei	10	-ve	-ve	-ve	-ve	13	-ve
Legionella pneumophila	13	-ve	-ve	-ve	-ve	40	5
Yersinia pestis	17	-ve	-ve	-ve	-ve	45	10
Moraxella catarrhalis	11	-ve	-ve	-ve	-ve	20	10
Hafnia alvei	7	-ve	15	-ve	7	8	10
Enterococcus faecalis	15	-ve	15	-ve	20	6	-ve

(-ve), negative result

Antibacterial activity of AgNPs synthesized by *S. platensis*

The antibacterial activity of biosynthesized silver nano-particle was performed against both gram positive bacteria as mentioned before by well diffusion method. The zone of inhibition of *S. platensis* extracts against bacteria was ranged between 5 mm to 45 mm at 200 µl/well. The nano material extract of *S. platensis* showed the highest mean zone of inhibition (30mm) against the gram positive cocci *Staphylococcus aureus*, followed by *Enterococcus faecalis* (6mm), in comparison to nano material + water + DMSO.

For Gram negative bacteria, the maximum zone of inhibition was recorded also in nano material extracts of *S. platensis* against *Yersinia pestis* (45mm) followed by *Legionella pneumophila* (40mm), both *Moraxella catarrhalis* and *Serratia liquefaciens* (20mm), *Shigella sonnei* (13mm), *Pseudomonas aeruginosa* (12mm), *Klebsiella pneumoniae* (11mm), *Enterobacter aerogenes* (10mm), and *Hafnia alvei* (8mm).

By increasing the volume of AgNPs synthesized *S. platensis* to 100 µL/well the zone of inhibition increases for both gram positive and negative bacteria. However, a Silver Nano particle has showed antibacterial activities more than Na-citrate solvent. This study in agreement with **Theivasanthi and Alagar (2011)** that reported Silver nano particles material have showed high antibacterial activities in comparison with other extracts.

The efficiency of the biosynthesized nano-material against the tested bacteria could be attributed to the adherence of small sized nano-

material to the bacterial cell membrane surface and thus disturbing its permeability and respiration functions **(Jagtap and Bapat, 2013).**

The utilization of *S. platensis* as antibacterial reagents or as nano material solution has various advantages like easy cultivation and availability. This biological method approach toward the synthesis of nano-material has numerous benefits, that is, non-toxicity, cost effectiveness, rapid reduction, and economic viability. Future prospects of this research would be large scale production of nano material using *S. platensis* and ascertaining its effectiveness against a broad spectrum of microbial populations. In addition to other investigations that will coverage of the *S. Platensis* effectiveness to synthesis silver nano-particles.

4. CONCLUSION

Synthesis of *S. platensis* algae as nano-material using organic solvents was carried out and tested for its antibacterial activity against pathogenic bacteria. It was clear that Na-citrate extract showed maximum zone of inhibition against all the bacterial while hexane extract of *S. platensis* showed minimum inhibition zone against bacterial pathogens. Nano material recorded the highest zone of inhibition against *Yersinia pestis* (45mm) in comparison with organic solvent extract (Na-citrate) against *Staphylococcus aureus* (20mm). *S. platensis* should be considered as an economic antibacterial agent than using medical antimicrobials against pathogenic bacteria. Other Future prospects of this research would be large scale production of AgNPs using *S. platensis* and at different concentrations in order to explore more benefits of *S. platensis* as bio-antibacterial for pathogenic bacteria.

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الأنشطة الميكروبية للسبيرولينا بلاتنيس كمضاد حيوي وكمادة نانونية لبعض عزلات البكتيريا المسببة للأمراض

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تتمتع السبيرولينا مثل العديد من أنواع البكتيريا الزرقاء الأخرى بالقدرة على إنتاج عدد كبير من المواد المضادة للميكروبات وخاصة الممرضة منها، لذلك فهي تعتبر كائنات حية مناسبة تماماً للاستغلال كعوامل للمكافحة الحيوية للبكتيريا المسببة للأمراض. في هذه الدراسة ، تم اختبار عدد من مستخلصات المذيبات العضوية المختلفة وخلطها مع المواد المضادة من الطحلب وكذا المواد النانوية المُصنعة من طحلب سبيرولينا بلاتنسيس ويرجع ذلك لنشاطها المضاد للبكتيريا وخاصة ضد البكتيريا المسببة للأمراض. حيث تم استخدام طريقة انتشار البئر القياسية لقياس الأنشطة المضادة للبكتيريا من خلال تنمية الطحلب في أطباق موللر هينتون أجار وتمت زراعته بمزارع الطحالب النقية من مسببات الأمراض البكتيرية في آجار مغذي عند 37 درجة مئوية لمدة 18–24 ساعة.

تم جمع عدد عشر عينات مياه بمحاذاة مصرف عمر بك ومن فرع دمياط لنهر النيل خلال الخريف (سبتمبر 2016) ، ثم تم عزل عدة أنواع من البكتيريا الممرضة موجبة جرام وسالبة جرام. ظهرت نتيجة مستخلص سيترات الصوديوم أقصى منطقة من التثبيط ضد كل أنواع البكتيريا الممرضة. في حين أظهر مستخلص الهكسان من سبيرولينا بلاتنسيس الحد الأدنى من منطقة التثبيط ضد مسببات الأمراض البكتيرية وذلك عند مقارنتها بمستخلصات المذيبات العضوية الأخرى. سجلت مادة النانو المُصنعة من الطحلب أعلى منطقة تثبيط ضد اليرسينيا الطاعونية (45 مم) مقارنة مع سترات الصوديوم ضد المكورات العنقودية الذهبية (20 مم) كمستخلص مذيب عضوي. ينبغي الأخذ في الاعتبار أن سبيرولينا بلاتنسيس يعتبر بمثابة مضاداً اقتصاديًا للبكتيريا بدلاً من استخدام مضادات الميكروبات الطبية ضد البكتيريا المسببة للأمراض.