Al-Mustansiriyah Journal of Science
ISSN: 1814-635X (print), ISSN: 2521-3520 (online)
Volume 29, Issue 3, 2018
DOI: http://doi.org/10.23851/mjs.v29i3.621

Research Article **Open Access**

Antimicrobial Activity of Freshwater Cyanobacterium Westiellopsis prolifica

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ArticleInfo

Received
05/10/2018

Accepted
20/10/2018

Published
10/03/2019

Abstract

The acetone and hexane of Westiellopsis prolifica extracts were examine efficiency against pathogenic bacterial and fungal isolates by using two methods: agar well diffusion and turbidiometric (tube method) against three Gram positive bacteria"Staphylococcus aureus, Bacillus subtilis, and Streptococcus sp." and three Gram- negative bacteria" Shigella sp., Proteus sp. and Pseudomonas aeruginosa " in additions to two isolates of fungi "Aspergillus niger and Candida albicans". The results showed that crude acetone extract for W. prolifica better than the haxane extract and more efficient on negative gram bacteria than positive gram bacteria. The results of the agar well diffusion method evaluated that W. Prolifica acetone extract has the highest antibacterial activities against Streptococcus sp., S. aureus and A.niger with an inhibition zone of (20) mm, and the inhibition diameter to other bacteria and fungi were between(15-10) mm.While tube method showed that the acetone extract exhibited the highest inhibition against A.niger and less inhibiting to C. albicans. Purification of the acetone extracts was made by silica gel column chromatography, and among the five groups extracts, Group 2 (Benzene 50ml) was selected and analyzed by GC-MS. The presence of main components identified in the extract as alcohols, acids, monoterpene eucalyptol, hydrocarbons (unidecane) aromaticslike, Para- Xylene and 1,2,3 trimethyl benzene, Phytol, n-Hexadecanoic acid, etc. These purified active compounds take part into broad horizons in the fields of biotechnology and pharmacy.

Keywords: Antimicrobial activity, Active compound, Cyanobacteria, Westiellopsis prolifica.

الخلاصة

تم فحص مستخلصات الأسيتون والهكسان لل *Westiellopsis prolifica* ضد العزلات البكتيرية والفطرية الممرضة باستخدام اثنين من الطرائق: النشر على الاطباق والعكورة (طريقة الأنابيب) ضد ثلاثة انواع بكتيرية موجبة لجرام " *Staphylococcus aureus*، *Bacillus subtilis* و *Streptococcus sp.*" وثلاثة سالبة لجرام "*Shigella sp.* ، *Proteus sp* و *Pseudomonas aeruginosa* " بالإضافة إلى عزلتين من الفطريات "*Aspergillus niger* و*Candida albicans*". وأظهرت النتائج ان مستخلص الأسيتون الخام لل *W. prolifica* أفضل من مستخلص الهكسان وأكثر كفاءة ضد البكتيريا السالبة لجرام من البكتيريا الايجابية لجرام. اظهرت نتائج طريقة الانتشار بالاجار أن مستخلص الأسيتون ل*Westiellopsis prolific* لديه أعلى فعالية ضد المسبحيات والمكورات العنقودية الذهبية وفطر *A .niger* بمنطقة تثبيط (٢٠) ملم، بينما كانت اقطار التثبيط لبقية انواع البكتيريا والفطريات تتراوح بين (١٠-١٥) ملم. وأظهرت طريقة ألانابيب أن مستخلص الأسيتون كان أعلى تثبيطا للفطر *A .niger*. وأقل تثبيطا لل *C. albicans*. تم إجراء تنقية لمستخلص الأسيتون باستخدام عمود هلام السيليكا، وتبين انه من بين خمس مجاميع استخلاص، تم اختيار المجموعة رقم ٢ (بنزين ٥٠مل) وتحليلها باستخدام جهاز GC-MS. المكونات الرئيسية في المستخلص شخصت على انها كحولات، احماض، monoterpene eucalyptol، هيدروكربونات (unidecane)، omaticslike ، Para- Xylene ، ١،٢،٣ بنزين ميثيل ، Phytol ،وحامض Hexadecanoic الخ. هذه المركبات الفعالة النقية قد يكون لها دور في فتح آفاق واسعة في مجالات التكنولوجيا الحيوية والصيدلة.

Introduction

The problem resistance of microbial to antibiotics is growing over time. The most important reason for the development of multidrug-resistant pathogens are the excessive use of antibiotics. So far there is microbial resistance to most antibiotics has been reported, additionally the side effects associated with antibiotics has increased the problem [1]. Therefore, there is a need to discover the new spectrum of antimicro-

bial agents which have minimal side effects, Emphasis has been placed on aquatic organisms, especially on cyanobactria. there are rich sources of Primary and Secondary Metabolites (Natural Products) and the most important for the pharmaceutical industry [2]. The secondary metabolites play a role in defense against either competitors or predators [3]. Cyanobacteria offer many characteristic for antimicrobial investigations due to their vast biodiversity and rapid growth rate [4]. Pathogenic bacteria tests were achieved by the aqueous and solvent extracts of this algae due to the existence of phenols, aliphatic compounds, terpenes, carbohydrates and fatty acids in this system, it posses antimicrobial character[5] The *Westiellopsis sp*. is the genus of the filamentous algae that belong to the Cyanophyta division, an investigation was to estimate the bioactivity of extracts of *Westiellopsis* sp. against Pathogenic bacteria. By Scanning Electron Microscopy (SEM) and GCM analysis were observed The morphological changes in bacteria and the chemical composition of the bioactive extract [6]. The aim of present study was to investigate the antimicrobial activity of hexane and acetone extract Westiellopsis prolific against pathogenic microbial by using two methods as agar well diffusion method and The turbid metric method (tube method). Then, *W. prolifica* extract was employed for GC/MS analysis, for detection of active constituents.

Materials and Methods

Sample collection and culture characterization

The cyanobacteria of *W.prolifica* isolated from the River Diyala in Nhrawan Baghdad city The isolates were identified using morphological variation studies and taxonomical approaches according to [7]. The sample was grown in BG-11 medium under condition (16h light \ 8h dark) at 25±2°C and 268µE/m²/s light intensity, the strain was harvested at their exponential phase of growth which is the 28th day [8].

Extraction of bioactive metabolites

According to method [9] with some modification,one gram of *W. prolifica* powder was extracted with 250ml of 95% acetone solvent using a Soxhlet extraction apparatus at 60 ºC for 3-4 h hours until the become the solvent colorless. The crude extract was dried by rotary evaporator at 40 ºC. Then the extract was weighted and stored at -20°C until further use.Repeat the same step using a hexane solvent.

FTIR Analysis

Fourier transforms infrared spectrometer based on [10] was used to analyze the samples, this analysis was carried out in the Department of Chemistry at Mustansiriya University. potassium bromide (KBr) powder was mixed with algal powder, The samples were analyzed in transmission mode at 400-4000 cm^{-1} wave number range.

Antimicrobial activity

The aceton and hexane of *W. prolifica* extracts were examined efficiency against pathogenic bacterial and fungal strains by using two methods as agar well diffusion method and The turbid metric method (tube method) [2]. *W. prolifica* extract was examined for their antibacterial activity were obtained from AL- Diwaniyah Teaching Hospital, against three are Gram- negative bacteria" *Proteus sp., Shigella sp. Pseudomonas aeruginosa*" three Gram positive bacteria" *Bacillus subtilis, Staphylococcus aureus, Streptococcus sp*." also, two isolates of fungi " *Candida albicans and Aspergills niger* ". Antimicrobial activity of extracts was estimated using the agar well diffusion method as described by[11]. The wells were filled with 100 µl of extract, and DMSO was used as negative control. Plates were incubated 24 hours at 37 ± 1 ºC for bacterial strains and 72 hours at 25 ± 2 ºC for fungal strains. The diameter of inhibition zones was measured in triplicates.The turbid metric assay was employed to evaluate the sensitivity of the test pathogen in liquid culture. In this assay of the 37C ^{o} for 24 h(bacteria) 28C ^{o} for 48 h (fungi) old culture (1ml) was inoculated in sterile nutrient broth (10 ml) and to this the 10^{-1}, 20^{-2}, 30^{-3}, 40^{-4}, and 10^{-5} dilutions of algae extract (0.5 ml) were added and allowed to incubate for 24 hrs at 37°C. The growth was measured spectrophotometrically at 600 nm in terms of turbidity of the

bacterial cultures. The readings were compared with that of the positive controls.

Fractionation crude extract of W. prolifica by solid adsorption chromatography

Acetone extract obtained from *W. prolifica* extraction of lyophilized biomass from a large scale culture of this strain was separated by silica gel chromatography Follow the method [12].

GC-MS analysis

Pure compound was subjected to GC/Mass investigation using (SHIMADZU GC/Mass QP 5050 A) instrument employing the following conditions: column: DB5, (Inert cap 1MS; 30 m × 0.25 mm id × 0.25 μm film thickness) carrier gas: (1ml/min); injector temp. (280^{O}C) detector temp, while the initial column temperature was set at 100 °C. A 5 μL sample volume was injected into the column, the temperature was raised to 225 °C, ramp rate of 12.5 °C/min.(For four minutes). The oven temperature was then raised to 300 °C at a ramp rate of 7.5 °C/min (hold time 5 min) [13]. The compounds were identified via comparison of their mass with original standards and NIST library search.

Statistical Analysis

Data are given of four determinations as mean ± standard deviation (SD). Statistical analyses were performed using a one-way analysis of variance. variations were counting significant at P values < 0.05 [14].

Results and Discussion

FTIR Fourier Transform Infrared Spectroscopy analysis of W. prolifica crude extracts:

FTIR spectroscopy has been vastly used to supply information on a domain of vibrationally active functional groups (inclusive –CH2, C–O–C,O–H, N–H, C=O, =C–H and –CH3) in biological sample [15]. The main chemical of cells, which indicates elevated C=O absorption ranging between 1763-1712 cm^{-1} linked to the main chemical groups fatty acid existing on the cell walls Figure 1.

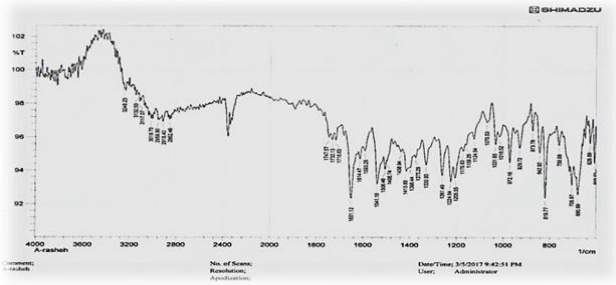

Figure 1:FTIR spectral of Crude extract *W. prolifica*

Antimicrobial activity by tube method

The crude extracts of *W. prolifica* have been used to determine the effect on the microbes by using a spectrophotometer and measured it at 600 nm of wavelength. In Table 1 showed significant differences in both crude extracts (acetone and hexane) when compared with the control group, In addition, It was found that the acetone extract was better tolerated against the bacteria and fungi under study than the hexane extract showed that acetone extract the highest inhibition in *A.niger* (0.263) nm and less inhibiting in C. *albicans* (0.330) nm.

Table 1. Antibacterial activity of intracellular extract of *W.prolifica* against microbe in600 wave length by tube method

Bacteria	Control (+ve)	Acetone	Hexane	LSD value
Staphylococcus aureus	0.742± 0.10	0.672± 0.08	0.506± 0.06	0.104 *
Streptococcus sp.	0.570± 0.06	0.549± 0.06	0.570 ± 0.06	0.094 NS
Bacillus subtilis	0.644± 0.11	0.633± 0.06	0.612± 0.08	0.117 NS
Proteus sp.	0.869± 0.14	0.669± 0.10	0.869 ± 0.14	0.109 *
Shigella sp.	0.713± 0.09	0.507± 0.03	0.238± 0.02	0.268 *
Pseudomonas aeruginosa	0.811± 0.12	0.472± 0.03	0.809± 0.09	0.183 *
Candida albicans	0.331± 0.03	0.330± 0.04	0.330 ± 0.04	0.064 NS
Aspergillus niger	0.710± 0.07	0.263± 0.04	0.673± 0.-07	0.153 *
*** (P<0.05), NS: Non-Significant.**				

In other hand the hexane extract, found that the highest inhibition of *Shigella sp.*(0.238) nm and less inhibiting *P.aeruginosa* (0.809) nm when compare with the control treatment, as well as showed no significant differences in both of crude extracts (acetone and hexane) on *Streptococcus sp., Bacillus subtilis* and *C. albicans* when compare with the control Figure 2.

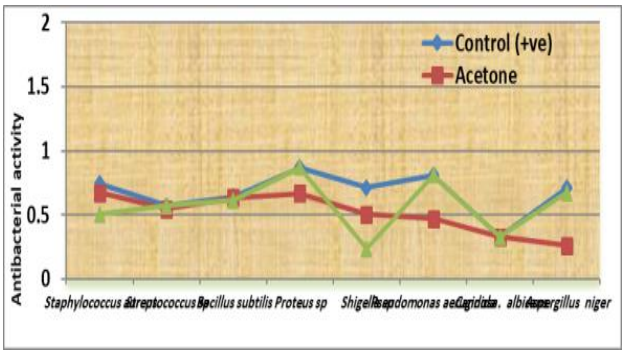

Figure 2:Antibacterial activity of intracellular extract of *Westiellopsis* against microbe in600 wave length by tube method

[16] illustrated that the optical density values obtained after treatment of the main bacterial (*S. aureus*, *P. aeruginosa*, *E. coli* and *Klebsiella* sp.) isolates form urogenital patient seminal. It showed the ethanol extracts of *Chroococcus sp* inhibition effects against *S. aureus* strains recorded (0.194 nm). Extracts of *W.prolifica* gained by different solvents show various degrees of antimicrobial activity due to that the antibiotic product dependent on the type of organic solvent [17]. Organic solvent provides more efficiency in extracting antimicrobial activity of the extract could be the presence of different chemicals such as flavonoids and triterpenoids besides phenolic that may affect growth and metabolism of bacteria. As well as concentration compounds and free hydroxyl group [18].

Antimicrobial activity by Agar well diffusion

The extract of *W.prolifica* was used to determine the antimicrobial effect of the agar well diffusion method. The results in Table 2 showed a significant difference in both extracts when compared with control. As well as revealed that the antibacterial effects of acetone extracts better than hexane extract Figure 3.

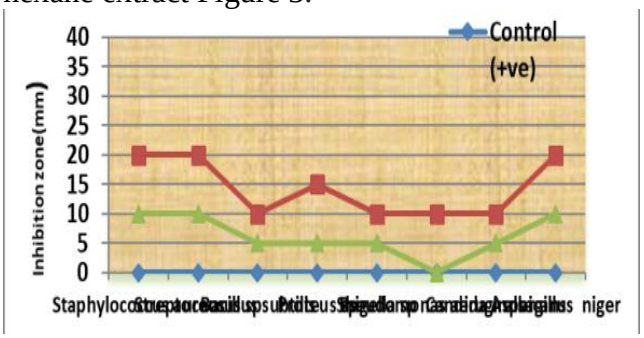

Figure 3:Inhibition zone (mm) of *W.profilica* extract against bacterial and fungi by Agar well diffusion assay

The result evaluated that *W. Prolifica* acetone extract has the highest antibacterial activities against *Streptococcus sp*., *S. aureus* and *A. niger* with the inhibition zones of (20) mm in addition the inhibition diameter Restrict between(15-10)mm with other bacteria and fungi.

Table 2:Inhibition zone (mm) of *W. pro*lifica extract against bacterial and fungi by Agar well diffusion assay

Bacteria	Control (+ve)	Acetone	Hexane	LSD value
Staphylococcus aureus	0 ± 0	20 ± 2.75	10 ± 1.61	5.26 *
Streptococcus sp.	0 ± 0	20 ± 2.75	10 ± 1.61	5.26 *
Bacillus subtilis	0 ± 0	10 ± 1.61	5 ± 0.39	4.94 *
Proteus sp.	0 ± 0	15 ± 2.43	5 ± 0.39	5.83 *
Shigella sp.	0 ± 0	10 ± 1.61	5 ± 0.39	4.94 *
Pseudomonas aeruginosa	0 ± 0	10 ± 1.61	0 ± 0	5.02 *
Candida albicans	0 ± 0	10 ± 1.61	5 ± 0.39	4.94 *
Aspergillus niger	0 ± 0	20 ± 2.75	10 ± 1.61	5.26 *
*** (P<0.05).**				

In this study, it was found that the active compounds found in the cyanobacteria extracts inhibit the bacteria in a varied and noticeable manner. This study is consistent with the principles of [19 and 20].

The inhibitory effectiveness of algae extract is different from that of fungi and bacteria, Where the mechanism of fungus differs in the cell wall structure than that found in the negative and positive Gram bacteria, In fungi the cell wall consists of the fungal glucan, chitin mannans and glycolproteins, whil the differences structure of the cell wall of both negative and positive Gram bacteria, The Gram negative bacteria cell wall is composed of lipoproteins and lipo polysaccharides, while Gram positive bacteria is composed of teichoic acids and peptidoglycan [18]. The mechanism of action of fatty acids, mainly affects on the membranes of cells where it leads to the entry of harmful components into the cell and reduces the absorption of nutrients and therefor affects on the cellular respiration, There is a variation in the results obtained by the researcher Due to several factors such as location and time of collection, culture media, growth stage [21].

The antimicrobial activity of extracts from microalgae is concerned to its lipids composition, attributed to palmitic acid, linolenic acid, oleic acid,And others [22].

Evaluation of the partially purified extract of *W.profilica*

Due to that the microlage extracts exist as a combination of different types of bioactive compounds with various polarities و Therefore, a separation process is required to identify and characterize the active compounds. The most prevalent way it is silica gel chromatography.

The results in Table 3 shows a significant difference in all groups of partially purified extracts against bacteria and fungi when compared with control. When refining purified crude extracts of some micro-algae species such as *Chroococcus sp.* should be utilized to obtain pure compounds and then used for the determination of structure and biological activity [23]. In this study focus on valuation of the purified acetone extract of *W.profilica* against bacteria and fungi using tube method.

The present study revealed that the purified intracellular and extracellular acetone extract to give five groups of purified as follows:**Group1**=Hexane 25ml +Benzene 25 ml; **Group2**= Benzene 50 ml; **Group3**=Ethyl acetate 25 m l+ Benzene 25 ml; **Group4**=Ethyl acetate 25 ml+ methanol 25ml and **Group5**=Methanol 50ml.

Chlorella vulgaris, *Nostoc Sp*. *Chlamydomonas sp* we obtain chemical compounds 'parsiguine, Ambigo A and B, chlorellin, Nostocin A and Nostocyclyne A' used as active anti-bacterial and fungal antibiotics [24].

Fatty acids,plays an important role in this system, it possess antimicrobial character these content varies depending on growth conditions and nutritional [23].

GC- Mass analysis of W. prolifica extracts

Thirty two chemical compounds were present and identified of *W. prolifica* as shown in Figure 4.

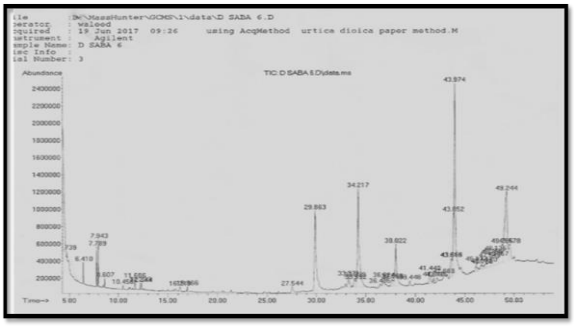

Figure 4: GC- Mass analysis of intracellular extracts

The highest area of the screened components was (20.14%) which belonged to fatty acid. The other compounds represented were Alcohols, acids, monoterpene Eucalyptol), hydrocarbons (undecane) and aromaticslike (Para- Xylene and 1,2,3 trimethyl benzene). There are several studies to purify the active compounds such as theThin layer chromatography (TLC)[25], also

anether studies reported the fractionation of the 4:1 methanol:water extract from Enteromorpha linza, using chloroform, Sephadex LH-20 gels and reverse-phase HPLC (high-performance liquid chromatography) using a C18 column to yield pure compounds [26].

Table (3). Antibacterial activity of purified intercellular extract of *W. prolifica* against bacteria and fungi by tube method

Microbial	Control	Group 1	Group 2	Group 3	Group 4	Group 5	LSD value
Staphylococcus aureus	0.742 ± 0.13	0.269 ± 0.03	0.358 ± 0.08	-	-	-	0.206 *
Streptococcus sp.	0.712 ± 0.09	0.349 ± 0.05	0.385 ± 0.06	-	-	0.077 ± 0.02	0.187 *
Bacillus subtilis	0.840 ± 0.13	0.407 ± 0.07	0.822 ± 0.11	-	0.440 ± 0.03	-	0.194 *
Proteus sp.	0.800 ± 0.11	0.166 ± 0.03	0.421 ± 0.07	0.340 ± 0.03	-	-	0.259 *
Shigella sp.	0.722 ± 0.08	0.423 ± 0.05	0.438 ± 0.08	-	0.188 ± 0.07	-	0.261 *
Pseudomonas. aeruginosa	0.730 ± 0.11	-	0.235 ± 0.03	-	-	-	0.198 *
Candida albicans	2.04 ± 0.34	0.958 ± 0.11	0.980 ± 0.08	0.633 ± 0.08	0.743 ± 0.11	0.334 ± 0.02	0.335 *

Conclusion

Partially purified (acetone and hexan) extracts had a higher efficacy than the crude extract against tested pathogenic microorganisms (bacteria and fungi). Saturated and unsaturated fatty acids and other biologically active compounds from the purified extracts (by Benzene 50 ml) exhibited efficient effect on gram negative bacteria and positive gram bacteria.

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