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Effects of green algae, *Chlorella Vulgaris*, extracts and its bioactive substances on the biofilm-producing microorganism

Ahmed Khayoon Abed Al- Abboodi*, Ghaidaa Husain Al- Rubiaee, Neihaya Heikmet Zaki

Department of Biology, College of Science, Al-Mustansiriya University, Iraq

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ABSTRACT

The current study aims at the *in-vitro* use of 2 types of extracts of *Chlorella Vulgaris*, one of the most widely known green algae species in the world, to evaluate their effects on biofilm-producing bacteria. In this study, 7 species of algae were isolated from different environments and then diagnosed according to the sources of green algae and greenish algae. Some of these isolates were proliferated under constant laboratory conditions at 25±2˚C, 200µm/m^2/sec, and 8:16 hours of light: dark using fixed farms. Then, these species were subjected to 2 types of polycarbonate-based solvents (hexane and chloroform). These extracts were tested on *E.Coli*, *Klebsiella*, *Acinetobacter*, *Serratia mesccens*, *St epidermis*, *S. aureus*, *Candida albicans*, and *Aspergills niger*. Using different methods of measuring turbidity of the red agar, micrometer plate, spreading Balacar, the effects of these extracts were examined on these microorganisms. Then, the algae were subjected to vertical chromatography to separate active substances. The hexane-based extract was more effective against *E. coli*, *Acinetobacter*, *St. aureus*. On the other hand, the chloroform-based extract showed slight effects on *Acinetobacter* and *C. albicans*. The best inhibitory activities were 30mm for *Staphylococcus aureus* and *Serratia* and 25mm for *E. coli*. The lowest inhibition activity was 15mm for *Klebsiella* and *C. albicans*. For the chloroform-based extract, the highest rate of inhibition was 20mm for *Klebsiella* and *S. aureus*. The lowest inhibition was 10mm for *Acinetobacter* and *C. albicans*. Separation method targeted the hexane-based extraction yielded hxan, gasoline group III ethyl estate, gasoline, group IV ethyl ester, methanol group, and methanol. The hexane + 25 ml of benzene and the fourth group of ethyl ester 25 + methanol showed higher inhibition activities against microbes than that using the other three groups. The hexane-based extracts and the related hexane + 25 ml of benzene and the fourth group of ethyl ester 25 + methanol of *C. vulgaris* were better than the chloroform-based extracts in their effectiveness against the studied microbes using the turbidity measurement method.

* Corresponding Author

Name: Ahmed Khayoon Abed Al- Abboodi
Phone: +9647814806483
Email: ahmedkhuyon@gmail.com

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INTRODUCTION

Infectious diseases are major causes of high rates of morbidities and mortalities in humans worldwide, in particular developing countries. The risk of diseases has increased widely in recent years due to heavy infections, and pathogenic bacteria have become resistant to common drugs due to random use of antibiotics. Antibiotic resistance in bacteria and fungi is one of the major health problems in the world. This issue has required the development of new alternatives to these drugs. Microalgae are rich sources of bioactive compounds

(Chowdhury *et al*., 2015). Secondary or primary metabolites related to these organisms may be potential bioactive compounds of interest that have industrial, therapeutic, and agricultural significance (Mugilan and Sivakami, 2016).

Microalgae exhibit a notable biodiversity; they can, in fact, be found as single cells, colonies, or extended filaments. They represent an essential role in the food chain in aquatic ecosystems; take up H_2O and CO_2 with the aid of solar energy to synthesize complex organic compounds, which are subsequently accumulated and/or secreted as primary or secondary metabolites (Al-Hashimi *et al*., 2016).

Microalgae grow under all environmental conditions such as fresh water brackish, the marine water of saline- hyper or hypo, acidic or alkaline, wide-range of temperatures and pH, and unique nutrient availabilities. Also, they have the adaptability to harsh environmental conditions such as heat, cold, drought, salinity, photo-oxidation, osmotic pressure, and UV exposure (Amaro *et al*., 2011).

This wide range of ecosystems contributes to countless chemical compounds that are able to be synthesized. Microalgae have been used for therapeutic purposes, and their systematic screening for bioactive effects began in the 1950s. In most microalgae, the bioactive compounds are accumulated in the biomass (Intercellular); however, these metabolites are excreted into the extracellular medium (de Morais *et al*., 2015). Several studies have shown that microalgae or their extracts have different biological activities including antitumor, antiprotozoal, antiviral, antioxidant, and cytotoxic activity against human-cancer cell-lines (Al-Rubaie *et al*., 2011; Elnabris *et al*., 2013; Al-Rubaie *et al*., 2014; Entesar, 2016).

The antimicrobial activity of microalgae has been attributed to compounds that belong to several chemical classes including indoles, terpenes, acetogenins, phenols, fatty acids, and volatile halogenated hydrocarbons (Amaro *et al*., 2011). The aims of this study were to identify the effects of 2 types of extracts of *Chlorella Vulgaris*, one of the most widely known green algae species in the world, to evaluate their effects on biofilm-producing bacteria.

MATERIALS AND METHODS

A sampling of microalgae

Clean glasses containers were washed using a method from (stein, 1973). During January to February of 2017, samples were collected from Al-Diwaniyah River, Al-Furat neighborhood, Al-Diwaniyah City, Iraq. The samples were collected using sterile container (100 ml) which is labeled with date and location of sampling. Then, the samples were transported immediately to a laboratory and then were incubated under suitable conditions (16hrs of light\ 8hrs of dark) at 25±2°C and 268 $\mu E/m^2/s$ light intensity (Malathi *et al*., 2015).

Algal isolation and purification

The collected samples were identified using classical algal classification references (Desikachary, 1959; Prescott, 1973). Algal isolation was conducted using two methods of streak plating (Sinigalliano *et al*., 2009; Stein and Borden, 1982; Prescott, 1982) and serial dilution methods (Stein and Borden, 1982).

Biomass and harvesting

Prepared flask contained 100ml Ch-10 culture media was used to transfer 25ml of isolated algae and then were incubated for 14 days. Then, this growth was transferred to 500ml of cultivating media and incubated again for 14 days. Then, it was transferred to1000ml of culture media. Finally, the growth was transmitted to glass pools of 4-L dimensions (50cm length, 40cm width, and 30cm high) to harvest biomass culture (Tredici, 2004; Falch *et al*., 1995). After 1 month, harvesting of the algae from biomass culture was done using centrifugation at 4000rpm for 10 minutes (Shelef *et al*., 1984). Then, the samples were washed, dried, weighed, and stored in 4°C (Jawad, 1982).

Organic extraction of *Chlorella Vulgaris*

According to Elnabris *et al*., (2013) with some modification, a method was followed to prepare crude extracts of algae as follows: 1g of *C.vulgaris* powder was exposed to 250ml of 95% hexane using a Soxhlet extraction apparatus at 60 °C for 3-4hrs. The crude extract was dried, collected, and stored at -20 °C until further use. Fourier transforms infrared spectrometer based on (Basniwal and Kaushik, 2014) was used to analyze the samples. This analysis was carried out in the Department of Chemistry, Mustansiriya University, Baghdad City, Iraq.

Microorganisms

Eight species of pathogenic bacteria and fungi were used which were 2 Gram-positive bacteria (*Staphylococcus aureus* and *Staphylococcus epidermis*), 4 Gram-negative bacteria (*E.coli*, *Klebsiella sp*., *Acinetobacter* sp., *Serratia macescens*), and 2 isolates of fungi (*Aspergills niger* and *Candida albicans*). These microorganisms were obtained from Al-Diwaniyah Teaching Hospital and Al-Kindy Teaching Hospital, Diwaniyah City, and Baghdad City respectively, Iraq.

Biofilm Production assays

Congo red test

Bacteria and fungi were isolated and incubated aerobically for 24-48hrs at 37˚C and 48-72hrs at 28˚C for bacteria and fungi respectively. The methods were followed from (Nivedith, *et al*, 2012).

Microtiter plate Method

Overnight bacteria were cultivated in brain-heart-infusion broth (BHIB) diluted 1:10. Then, amount of 20µl of bacteria was added to180µl BHIB, and fungi were cultivated in Sabouraud's Dextrose Broth in 3 days. The methods were adopted from (Holt *et al*., 1994; McWilliams *et al*., 2012).

Crude-extract-effect assays of *C.vulgaris* on biofilm formation

Congo red agar

Crude extract at 1ml was added to Congo red agar medium at room temperature to totally dry. The method was followed by (Blanco *et al*., 2005).

Microtiter plate method

The overnight bacterial culture was cultivated in BHIB, and fungi were cultivated in SDB. Bacterial and fungal suspensions and crude extracts of *C.vulgaris* at 100µl each were added to 96-well flat-bottomed microtiter plates. The method was followed by (Namasivayam *et al*. 2013). The microtitre-plate anti-biofilm assay estimated the percentage of fungal and bacterial biofilm reduction using the following equation,

$$\%\ \text{of Inhibition of biofilm formation} = \frac{\text{OD of(+ve) control} - \text{OD of treatment}}{\text{OD of(+ve)control} - \text{OD of(−ve)control}} \times 100$$

Antibacterial Production Assay

It was evaluated using turbid metric assay (tube method). It is based on inhibition the growth of microbial growth in a fluid medium containing uniform distribution of an antimicrobial compound. The readings were compared with positive controls. The agar-well-diffusion method uses antimicrobial and antifungal activities of crude extracts of *C. vulgaris* on a nutrient agar. The diameters of the inhibition zones were measured (Attaie *et al*., 1987).

Gas chromatography-Mass Spectrometry (GC-MS

For GC-MS analysis, a high-temperature column (Inert cap 1MS; 30 m × 0.25 mm id × 0.25 µm film thickness) was purchased from Agilent Technologies (SHIMADZU, Japan). The injector and detector temperatures were set at 280 ˚C while the initial column temperature was set at 100˚C. At first, 5µl of a sample was injected into the column and run using split (1:10) mode after 1min, and the oven temperature was leveled up to 225˚C at a ramp rate of 12.5˚C/min (hold time 4min). The oven temperature was then elevated to 300˚C at a ramp rate of 7.5˚C/min (hold time 5min). The helium carrier gas was programmed to maintain a constant flow rate of 17.5 ml/min, and the mass spectra were acquired and processed using both Agilent GC-Mass Solution (SHIMADZU, Japan) and a postrun software. The compounds were identified by comparing their masses with NIST library search and authentic standards. The resulted compound were tested on biofilm forming using the same methods that were mentioned above.

Statistical Analysis

The Statistical Analysis System- SAS (2012) software was used. Chi-square x^2 test was used to compare percentages and least significant differences. ANOVA (LSD test) was used to compare between means in this study.

RESULTS AND DISCUSSION

Identification of Algae

Seven different species of algae were identified. They included 3 species of Chlorophyta and 3 species of Cyanophyta as shown in table and figure 1. The present study was focused on *Chlorella vulgaris* from Chlorophyta.

The reason for such diversity of the isolated algae was due to the high level of water pollution which is rich in nutritious compounds such nitrogen, phosphorus, iron, and magnesium in addition to environmental factors that play a role in the growth of algae such as intensity of light, tempera- ture, pH, and others (Al-Hussieny and Abed Thijar,2016).

Determination of biofilm formation by Congo red agar

Figure 2 shows color-based detection of biofilm formation from red (negative) to black (positive) that were used as controls. Biofilms are complex mixtures of microbes which are predominantly attached to hard surfaces. This specific-adhesion pattern is mediated by bacterial-cell-wall structures, which are genetically determinants of bacterial species (Hammadi and Yousif, 2014). They are often enclosed by thick polysaccharide layer which makes them resistant to antibiotics and thus very hard to be eliminated (Kaiser *et al*., 2013, Lu, *et al*., 2014).

Evaluation of the anti-biofilm activity of *C.vulgaris* extract

Congo red Agar

Table 2 reveals that the hexane-based crude-extract of *C.vulgaris* was better than the chloroform extract in the anti-biofilm formation at 50µl.

Table 1: The seven species of algae that have been isolated from different locations

Division	Class	Order	Family	Genus	Species
Chloro-phyta	Chloro-phyceae	Chlamydo-monadales	Chlamydo-monadaceae	*Chlamydomo-nas*	*Chlamydomonas sp*
	Zygnema-tophyceae	Zygnematales	Zygnema-taceae	*Zygnema*	*Zygnema cyano-sporum*
	Zygnema-tophyceae	Zygnematales	Zygnema-taceae	*Spirogyra*	*Spirogyra sp.*
	Chloro-phyceae	Chlorellales	Chlorellaceae	*Chlorella*	*Chlorella Vul-garis*
Cyano-bacteria	Cyanophyceae	Stigonematales	Hapalosi-phonaceae	*Westiellopsis*	*Westiellopsis prolifica*
		Nostocales	Oscillatoria-ceae	*Oscillatoria*	*Oscillatoria For-mosa*
		Nostocales	Oscillatoria-ceae	*Oscillatoria*	*Oscillatoria amoena*

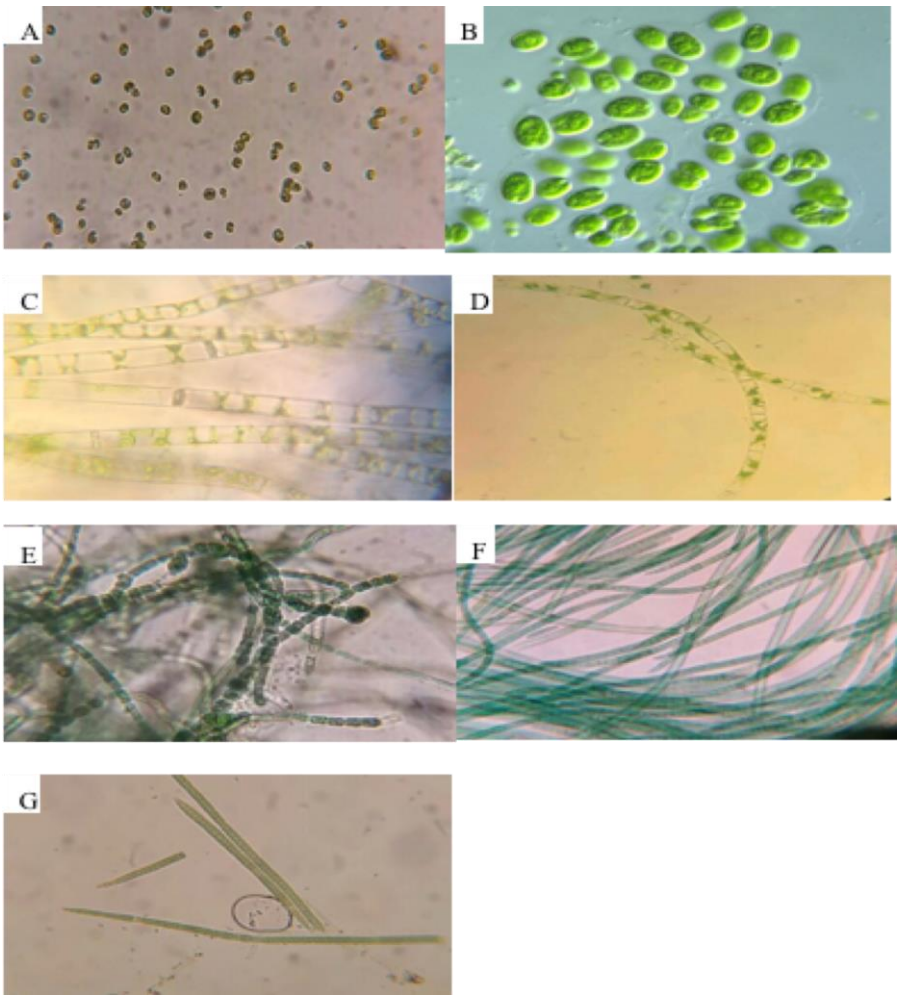

Figure 1: Isolated microalgae. A. Chlorella vulgaris B. Chlamydomonas sp C. Spirogyra sp D. Zygnema cyanosporum E. Westiellopsis prolifica F. Oscillatoria amoena G. Oscillatoria Formosa

The extract of *Chlorella vulgaris* was effective against biofilm formation for all strains especially gram-negative bacteria and more specifically against *E. coli* and *Acinetobacter* (+++) while the rest of the strains showed moderate activity (+). On the other hand, the chloroform-based extract revealed that anti-biofilm activity was strong against *Acinetobacter*, *Serratia marcescens*, and *C. albicans* (++). While the rest of the strains showed moderate activity (+), Figure 3 which explains the pink colonies in the presence of cellular extract indicating a loss of biofilm-forming ability in all bacterial and fungal isolates of the current study. This loss was probably due to exo-polysaccharides are not produced in a medium that contains cellular extract of the algae. Black colonies indicate biofilm appearance (Raulio, 2010).

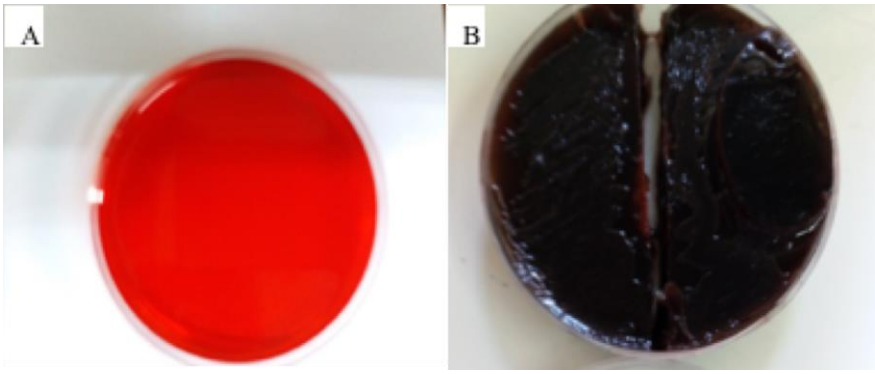

Figure 3: Biofilm production on Congo red Agar. A- Control negative (-). B- Control positive (+)

Table 2: The Anti-biofilm activity of crude extract of *Chlorella vulgaris* on Congo red agar

Bacteria	Anti-biofilm activity of the extract	
	hexane	chloroform
Escherichia coli	+++	+
Klebsiella pneumonia	+	+
Acinetobacter baumannii	+++	++
Serratia macescens	+	++
Staphylococcus epidermis	+	+
Staphylococcus aureus	+	trace
Candida albicans	+	++
Aspergillus niger	+	trace

Trace=very weak; += Moderate; ++ = strong; +++= very strong

Figure 2: the Anti-biofilm activity of the crude extract of *C.vulgaris* at 50µl via Congo red agar. (A) Acinetobacter (B) Serratia marcescens (C) *C.albicans*

Anti-biofilm activity via microtiter-plate method

Table 3 shows a significant difference between both hexanes- and chloroform-based extractions for all treatment via microtiter-plate method at100µl. The effect of a crude-hexane-based extract of *C. vulgaris* was more efficient than the chloroform-based extract for bacteria and fungi. The highest biofilm inhibition in the hexane-based extract reached 19% against *S. marcescens* and *A. niger* while the lowest biofilm inhibition was against *E.coli*, 76%. On the other hand, the highest biofilm inhibition regarding the chloroform-based extract recorded 22% against *A. niger*, and the lowest biofilm inhibition was against *Klebsiella* sp, 97%, figure 4.

The current results of *C. vulgaris* extract efficiently affect the size and thickness of the biofilm and significantly reducing the resistance of the pathogenic bacterial cells. In this case, the extracellular polymeric substances (EPS) from microbial production play a major role in the strong adhesion and development of the rigidity of biofilm (Mah *et al*., 2003).

EPS provides increased the resistance of the bacteria against antibiotics and harsh environmental conditions (Annuk *et al*., 1999). So, reduction in the EPS will lead to easy exposure of the bacterial cells to antimicrobial compounds and reduction of bacterial biofilm. Reduced adhesion between the bacterial cells will affect the total count of bacterial population dwelling inside a biofilm matrix which will also support the inhibition of bacterial biofilm. Hexane-based extraction was more effective than chloroform-based extraction against biofilm due to the presence of short-chain fatty acids, methyl lactic acids, and butanoic acid.

Evaluation of antimicrobial activity of *C.vulgaris*-crude extract

Tube method

Table 4 shows significant differences between crude extracts, hexane-and chloroform-based extractions when compared with the control group. In addition, there are no significant differences for both extracts on *S. epidermis*, *Serratia sp*, and *C. albicans*. It is found that the hexane-based extract was better against the bacteria and fungi than the chloroform-based extract. It also reveals efficiency on Gram-negative bacteria better than that on Gram-positive bacteria and fungi, figure 5.

Table 3: The percentage effects of crude extract of *C.vulgaris* on biofilm

Microbes	Control	*Hexane*	*Chloroform*	Chi-Square (χ^2)
Escherichia coli	100	76%	86%	
Klebsiella pneumonia ,	100	69%	97%	8.516 **
Acinetobacter baumannii	100	74%	65%	8.931 **
Serratia macescens	100	19%	39%	12.563 **
Staphylococcus epidermis	100	50%	81%	10.406 **
Staphylococcus aureus	100	55%	88%	10.672 **
Candida albicans	100	22%	54%	12.984 **
Aspergillus niger	100	19%	22%	14.702 **
Chi-Square (χ^2)	--	12.827**	14.092 **	--

** (p<0.01)

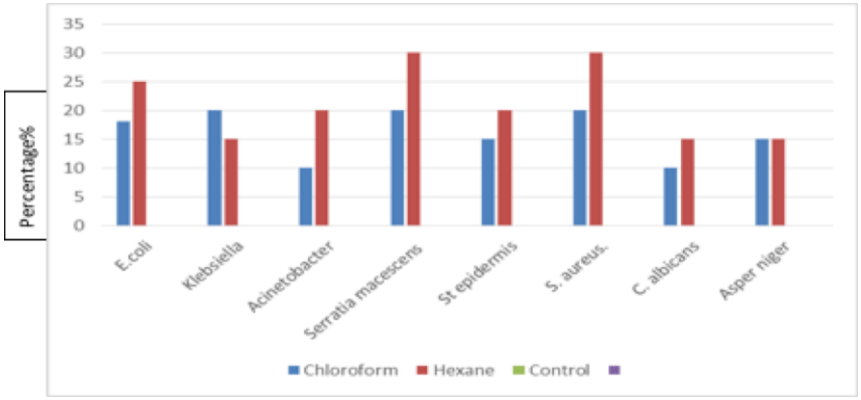

Figure 4: The percentage effects of *C.vulgaris* extracts on biofilm

Table 4: Antibacterial activity of curd extract of *chlorella vulgaris* against pathogenic microorganisms at 600nm wavelength via tube method

Bacteria	Control (+ve)	Hexane	Chloroform	LSD value
Escherichia coli	1.069	0.256	0.66	0.472 **
Klebsiella pneumonia	1.139	0.111	1.103	0.657 **
Acinetobacter baumannii	0.918	0.139	0.864	0.498 **
Serratia macescens	0.570	-	0.549	0.376 NS
Staphylococcus epidermis	0.644	0.612	0.633	0.228 NS
Staphylococcus aureus.	0.439	0.034	0.349	0.277 **
Candida albicans	0.320	-	0.312	0.169 NS
Aspergillus niger	0.710	0.673	0.263	0.305 **
LSD value	0.309 **	0.288 **	0.631 **	--

** (p<0.01); NS: Not significant.

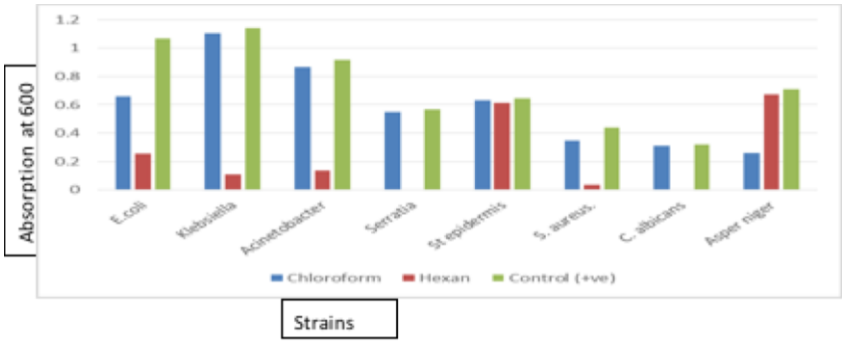

Figure 5: Antibacterial activities of *C.vulgaris* extracts against microorganisms at 600nm via tube method

Agar well diffusion method

Table 5 shows a significant difference in both crude extracts and between strains for all treatment when compared with the control. They revealed that the antibacterial effects of hexane-based extracts were better than that using chloroform-based extract. It had the highest antibacterial activities against *S. aureus* and *Serratia* with an inhibition zone of 30mm. It had the lowest effects against *Klebsiella* and both studied fungi, 15mm, figure 6.

Chemicals that include alkanes, flavonoids, and triterpenoids and phenolic compounds may affect

Table 5: Antimicrobial activity of *C. vulgaris* crude extracts as presented by inhibition zone diameter (mm).

Microorganisms	inhibition zone (mm)			
	Control	Hexane	Chloroform	Chi-Square (χ^2)
E.coli	-	25	18	8.612 **
Klebsiella sp.	-	15	20	7.157 **
Acinetobacter	-	20	10	7.361 **
Serratia marcescens	-	30	20	9.473 **
St epidermis	-	20	15	7.252 **
S. aureus.	-	30	20	9.473 **
C. albicans	-	15	10	6.013 **
A. niger	-	15	15	6.284 **
Chi-Square (χ^2)	--	6.926 **	2.073 NS	--

** (p<0.01); NS: Not significant.

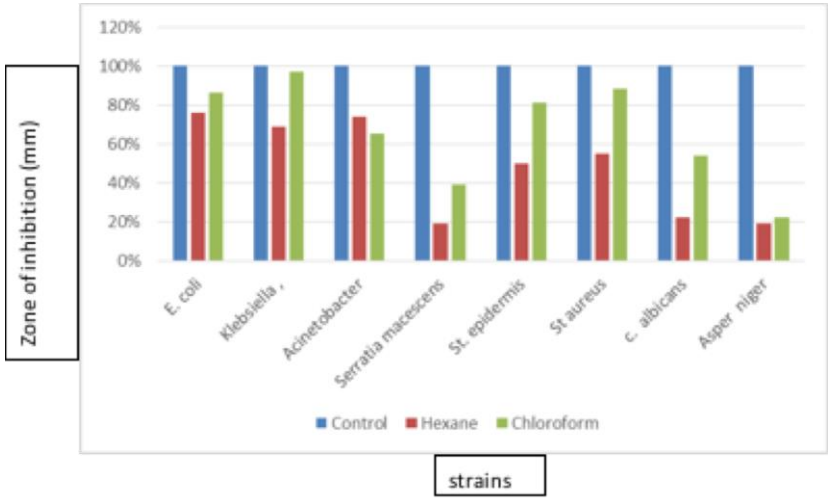

Figure 6: Inhibition zone (mm) of *C. vulgaris* extract against bacteria and fungi via agar well diffusion assay

growth and metabolism of bacteria. Also, they could have an activation or inhibition effects on microbial growth according to their constituents and concentrations of free hydroxyl group amides and alkaloids (Ghasemi *et al*., 2004).

The antimicrobial activity of extracts from microalgae was related to their lipid composition. It has been attributed to γ-linolenic acid, eicosapentaenoic acid, hexadecatrienoic acid, docosahexaenoic acid, palmitoleic acid, lauric acid, oleic acid, lactic acid, and arachidonic acid (Amaro *et al*., 2011; Smith *et al*., 2010). The mechanism of action of fatty acids affects various structures in microorganisms; however, cell membranes are the most impacted. Membrane damage most likely leads to a loss of internal substances from the cells, and the entry of harmful components reduces nutrient absorption in addition to inhibiting cellular respiration. The ability of fatty acids to interfere with bacterial growth depends on both their chain length and the degree of unsaturation. Fatty acids with more than 10 carbon atoms apparently induce lysis of bacterial protoplasts (De Morais *et al*., 2015).

Separation method targeted the hexane-based extraction yielded hand, gasoline group III ethyl estate, gasoline, group IV ethyl ester, methanol group, and methanol. The hexane + 25 ml of benzene and the fourth group of ethyl ester 25 + methanol showed higher inhibition activities against microbes than that using the other three groups.

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