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Full Length Research Paper

Antibacterial activities of the extracts of cyanobacteria and green algae isolated from desert soil in Riyadh, Kingdom of Saudi Arabia

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In compliance to the recent surveys on algal species and their potentials to produce biologically active compounds, seven algal species belonging to cyanobacteria such as *Spirulina platensis, Nostoc linckia, Phormidium autumnale, Tolypothrix distorta* and *Microcystis aeruginosa* and green algae such as *Chlorella vulgaris,* and *Dunaliella salina*, were isolated from soil in Riyadh, Saudi Arabia. All algal species were morphologically identified using scanning electron microscopy, in addition to light microscopic analysis. Liquid media BG11 was used for cultivation of algal isolates for four weeks; chlorophylls were estimated in the exponential growth phase after two weeks growth in liquid BG11 medium; in liquid medium after four weeks incubation, maximum growth was recorded for *Dunaliella salina* and *N. linckia* using cell mass weight. Extraction of algal species were tested for antibacterial activity using agar well diffusion method against a variety of human pathogenic bacteria such as *Salmonella suis* ATCC 13076; *Pseudomonas aeruginosa* ATCC 27583; *Escherichia coli* ATCC 25922; *Staphylococcus aureus* ATCC 25923; *Bacillus subtilis* ATCC 6633; *Shigella sonnei* ATCC 11060 and one yeast *Candida albicans* ATCC 10231. The bioactive metabolites from algal species were extracted using three different solvent system as methanol: acetone: diethyl ether (5:2:1), acetone and ethanol. The methanol : acetone : diethyl ether solvent system was the most effective solvent system among the three and showed strong activity against *S. sonnei*, *S. aureus,* and *B. subtilis*. Ethanol extract of *P. autumnale* showed activity against only *C. albicans*.

Key words: Algae isolation, scanning electron microscope, cyanobacteria, green algae, algal extract, antimicrobial activity, human pathogenic bacteria and yeast.

INTRODUCTION

Active ingredients derived from algae species have been studied in the last decade. A wide variety of beneficial applications could be obtained from algae species including marine and soil origins; the most studied ones are those which have been known to generate beneficial applications for human, attaining health maintenance (Stewart et al., 2006; Kay, 1991; Belay et al., 1993; Hayashi et al., 1994; Qureshi et al., 1996). In the mean time, a wide range of studies have been performed addressing this enquiry. One of the most important values of active compounds extracted from algae is it's biological control on microbes that have been identified pathogenic to human (Zulpa et al., 2003; Abedin et al., 2008). Our goal is to explore an efficient compounds of antimicrobial effect, especially those derived from natural sources in Saudi Arabia, in order to diminish side effects of antibiotics (Febles et al., 1995; Sethubathi et al., 2010; Kulik, 1995), as the latter could raise toxicities and serious threatened conditions in some cases referring to recent reports of WHO, on the other hand, microbial resistance to antibiotics has been developed lately in health institutions.

Many studies have been established to prove the antimicrobial effect of metabolites extracted from algal species especially those derived from blue green algae

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(Zulpa et al., 2003; Abedin et al., 2008; Kulik, 1995). A wide variety of Gram positive, Gram negative bacteria and fungi, were reported to be affected with these compounds (Febles et al., 1995; Sethubathi et al., 2010). These findings are now opening new trends in pharmaceutical industry, as the chemical structure of various biological active extract have been revealed in many studies (Masuda et al., 1997; Rosell et al., 1987). In the present study, different algal extract using three different solvent systems (methanol: acetone: diethyl ether, acetone and ethanol) from different species was tested for their activities against seven human pathogenic bacteria *in vitro*.

MATERIALS AND METHODS

Sample preparation

Isolation and cultivation of algal species

Seven algal strains belonging to cyanobacteria and green algae (*C. vulgaris*; *S. platensis*; *N. linckia*; *P. autumnale*; *D. salina; T. distorta* and *M. aeruginosa*), obtained from the Phycology Laboratory, Botany Department, Faculty of Science, King Saud University, Saudi Arabia were selected for screening of their antimicrobial activity. Algal species were isolated from different soils in Saudi Arabia according to standardized algae isolation procedure as follows; 10 g of soil samples were placed in 250 ml Erlenmeyer flasks each containing 100 ml of the sterile medium such as; BG11 (Rippka et al., 1979), Z8 (Kotai, 1972) and Chu's no 10 (Gerloff et al., 1950) and left under the favourable growth conditions. Any colored growth was picked up, subcultured and streaked several times in a solidified agar medium plates (solidification is performed by adding 15 g/L of agar-agar added to the liquid medium). Successive transfers from liquid to solid medium and re-subculturing yield uni-algal cultures (Rippka et al., 1979; Vaara et al., 1979). Further identification was performed by scanning electron microscopy in addition to botanical approach (light microscopic morphology). Each isolate was subcultured within BG11 media following composition in grams per liter of distilled water: 1500 mg $NaNO_3$; 40 mg K_2HPO_4; 75 mg $MgSO_4.7H_2O$; 36 g $CaCl_2.2H_2O$; 6 mg citric acid; 20 mg Na_2CO_3; 1 mg Na_2EDTA; 630 mg ferric ammonium citrate; Trace-metal mix, 1 ml/lit. Trace-metal were included: 2.86 g H_3BO_3; 1.81 g $MnCl_2.4H_2O$; 0.222 g $ZnSO_4.7H_2O$; 0.39 g $Na_2MoO_4.2H_2O$; 0.079g $CuSO_4.5H_2O$; 0.0494 g $Co(NO_3)_2.6H_2O$. After autoclaving and cooling, pH was adjusted to 7.1) (Rippka et al., 1979). All algal cultures were allowed to flourish at 20 to 30°C under constant light for four weeks. Algal cells were collected in the exponential growth phase by filtration for solvent extraction.

Biomass estimation

The cultures were harvested and known quantity of cells was washed thrice with distilled water after centrifugation at 5000 rpm. Then, the pellet was filtered and incubated overnight at 60°C. The percentage of dry weight of algal biomass was estimated.

Pigments estimation

A known quantity of algal biomass was homogenized and extracted with 80% acetone. The pooled extract was incubated overnight at –20°C, and chlorophylls content was estimated using Lichtenthaler method (Lichtenthaler, 1987).

Scanning electron microscopy

For the purpose of assessing morphological characteristics of the selected cyanobacteria and green algae species in this study, scanning electron microscopy studies were carried out. In order to scan the samples and spore morphology using the scanning electron microscope (SEM), the agar containing the samples were first cut using sterilized straw and then soaked overnight in 1% osmium tetroxide. Then, all the samples were washed one by one with sterile distilled water three times with 5 min interval from each wash. Gradually, dehydration technique was used to remove any water from the agar samples using low to high concentration of ethanol series (50 to 100%). Then, ethanol dried agar samples were soaked in hexamethyldisilazane ($C_8H_{18}NSi_2$) (Spi supplies, USA) for 2 h at room temperature. The soaked samples then dried on a clean filter paper over night and finally gold coated by sputter gold coater and then samples were attached on the aluminium sample stub by using tape and then viewed with the scanning electron microscope (SEM, JEOL, JSM, 3060LV) (Ismet, 2003; Ismet et al., 2003, 2002; Tamura et al., 1994).

Algae extractions

For organic extract, algae biomass was mixed with methanol:acetone:diethyl ether using 5:2:1 volumes respectively, and shaken for 3 days at 30°C in a shaker incubator. The mixture was separated by filtration using filter paper. Then, the combined aqueous extracts were evaporated to dryness and the residue re-dissolved in 2 ml distilled water to form stock solution (50 mg/ml). Similar extraction process was followed with the two other organic solvents, acetone and ethanol.

Bacterial bioassay

Microbial indicators and growth conditions

Seven microorganisms including Gram positive, Gram negative bacteria and yeast were used in this study; *S. suis* ATCC 13076, *P. aeruginosa* ATCC 27583, *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, *S. sonnei* ATCC 11060 and *C. albicans* ATCC 10231. Bacterial strains and yeast were kindly provided by Dr. Ismet Ara from Bacteriology Laboratory, Botany Microbiology and Department, Faculty of Science, King Saud University, Saudi Arabia. Antibacterial activity were performed using agar well diffusion method with three types of algal extraction and bacterial cultures were obtained by inoculating the strains on nutrient agar plates and incubated at 37°C for 24 h. Colonies of freshly inoculated bacteria were added into nutrient broth and incubated overnight at 37°C (bacterial turbidity adjusted to 0.5 McFarland standard was obtained). For the bioassay, bacterial colonies were spread using sterile cotton swab onto Muller Hinton agar plates.

The plates were allowed to dry, and then distant wells were made within agar to be loaded with the organic extracts.

Testing antimicrobial activity by the agar-well diffusion method

The antagonistic activity of algae extracts was determined using cut–diffusion technique in which cut (5 mm) was punched upon the surface of agar plates previously inoculated with each of the above mentioned indicator strains. Each well bottom was sealed with two

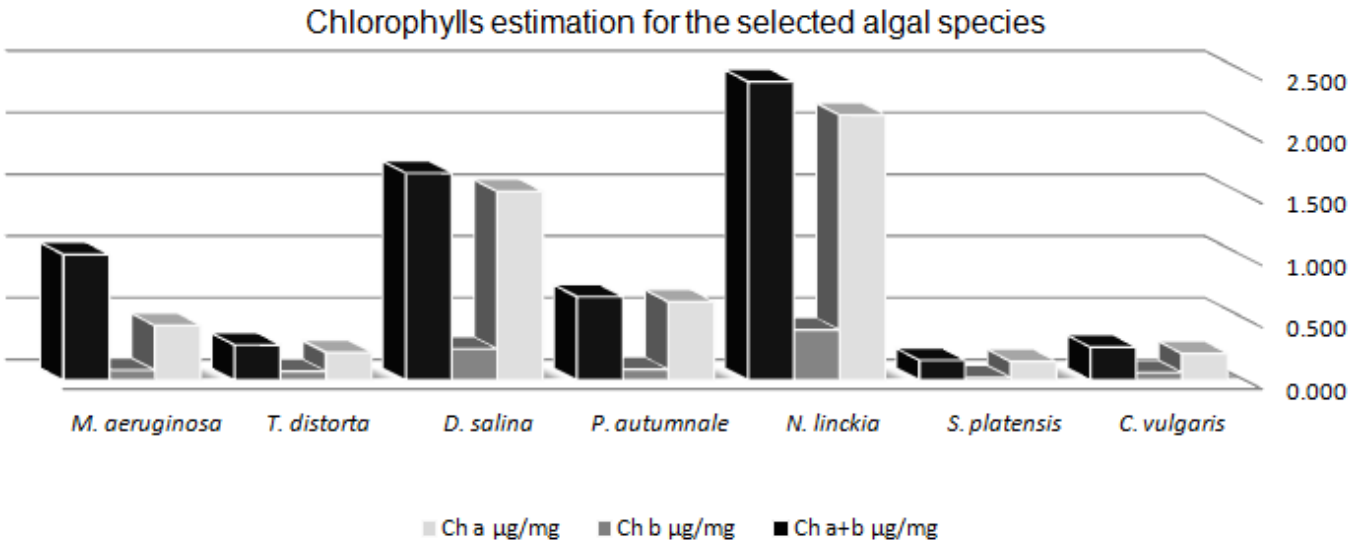

Figure 1. Quantification of chlorophyll a and b using acetone as extraction solvent subjected to the microalgae species. Maximum growth was recorded for *D. salina* and *N. linckia,* recording chlorophyll a+b µg/mg as 1.675 and 2.415 µg/mg, respectively.

drops of sterile water agar. About 100 µl of algal extract were transferred into each well. Wells loaded with the extracting solvents were used as controls; the plates inoculated with bacteria were incubated at 37°C for 24 h. After incubation, the diameter of the inhibition zone was measured with calipers and the results were recorded in mm (data not shown).

RESULTS

Scanning electron microscopy

Scanning electron microscopic analysis was performed for the algae cultures in order to illustrate genus and species of algae used in this study (Figure 2). Thus, isolation, purification and identification up to species level showed the following species: *C. vulgaris*; *S. platensis*; *N. linckia*; *P. autumnale*; *D. salina; T. distorta* and *M. aeruginosa* (Figure 1).

Growth indicator of algal species

The results presented in Figure 1 reveal pigment content of algal species in the exponential growth phase (after four weeks incubation). The maximum growth was recorded for *D. salina* and *N. linckia,* referring to the measures of chlorophylls a + b µg/mg (Figure 1).

Antimicrobial activities of different algal extracts

The resulted algae extracts were assayed against four human pathogenic Gram negative, two Gram positive bacteria, and one yeast strain. Table 1 shows the effects of the selected algae extracts isolated from soil and shows that methanol: acetone: diethylether extracts notably inhibited different kinds of targeted species in this study and mostly affected *S. aureus*, *B. subtilis* and *S. sonnei* (Table 1). *S. sonnei* showed inhibition with all the algae species used in this study, with the widest inhibition zone (about 25 mm) by *P. autumnale* (Figure 3A). A moderate inhibition (about 18 mm) of *C. albicans* by *P. autumnale* extract was detected with the ethanol extract. At the same time methanol: acetone: diethyl ether extract *of P. autumnale* and *T. distorta* showed good activity (~20-25 mm) against *S. aureus* and *B. subtilis*. In comparison with methanol: acetone: diethyl ether and ethanol extracts, weak activity (~8-15 mm) was recorded with acetone extract. Moreover, very weak inhibition of *P. aeruginosa* by *D. salina* was detected with inhibition zone of 8.5 mm. In this study, methanol and acetone were not used as bactericides when used as control and there were no remains in the crude extracts of these materials.

DISCUSSION

Our goal in this study is to assess the biological production of bioactive compounds by some cyanobacteria and green algae species isolated from natural sources in Saudi Arabia. The isolated species in this study showed similarity with that found at world deserts where fluctuation in temperature and humidity conditions predominate (Wierzchos et al., 2006; Bhatnagar et al., 2005; Jafari et al., 2004; Rios et al., 2004; Pichel et al., 2001).

This study confirms that the methanol: acetone: diethyl ether extract of different cyanobacteria and green algae

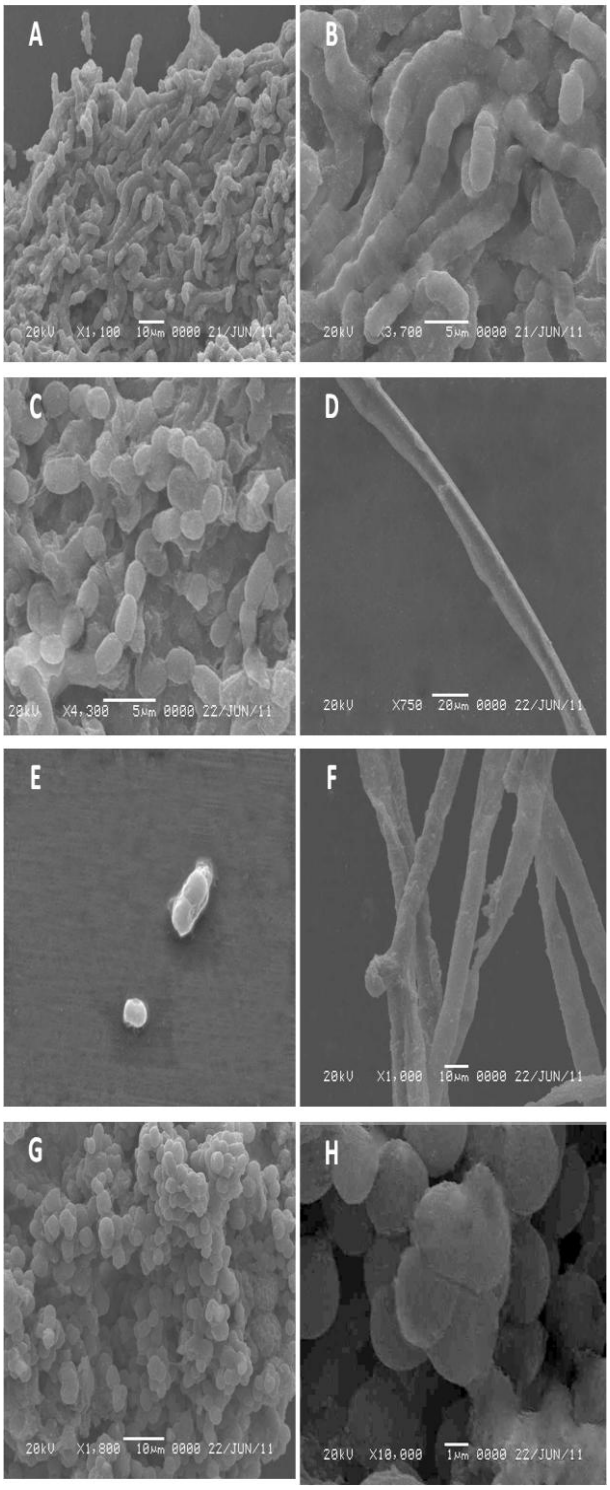

Figure 2. Morphological identification using scanning electron microscopic examination of *S. platensis* (A, B), *N. linckia* (C), *P. autumnale* (D), *D. salina* (E), *T. distorta* (F), and *M. aeruginosa* (G, H).

species have significant activity against some of the tested human pathogenic microorganism. As shown in the results, the affected pathogenic bacterial strains were not equally susceptible to the bioactive metabolites produced from algal species; this could be caused by the phylogeny of the microorganism strain (Philip et al., 2009). In correlation to Figure 1, no relation between algal growth indicators and its' intra-specific variability to produce active metabolites was established in this study. On the other hand, a significant difference in antimicrobial activity was noticed between different extracts within some algal species, suggesting that the efficiency of algal bioactivity depends on the type of solvents used in extraction, in addition to the type of algal species mainly (Mian et al., 2003; Salvador et al., 2007).

In this study, extract with combination of the three solvents methanol, acetone and diethyl ether, gave the highest bioactivity among acetone and ethanol extracts, in contrast to previous studies, all these solvents showed the most pronounced inhibitory effect against Gram positive, Gram negative bacteria and yeast (Umamaheshwari et al., 2009; Patil et al., 2011). Antimicrobial activity of methanolic extracts made from blue green and green algae have been reported in many studies (Ostensvik et al., 1998; De Mule et al., 1991; Ishida et al., 1997). It was reported that *P. aeruginosa*, *E. coli*, *B. cereus*, *P. vulgaris* have bioactivity against some fungi species (Prashantkumar et al., 2006). Furthermore, acetone and diethyl ether have been proved to be the best solvent for extracting antimicrobial agents from algae especially cyanobacterial species (Moreau et. al., 1988; Ozdemir et al., 2004). All of the previous investigations mentioned above go in harmony with those obtained with methanol: acetone: diethyl ether extract in our study, where all the studied algae gave inhibitory effect toward one of the targeted bacteria at minimum, with *T. distorta* having the most effective activity against *S. aureus*, *B. subtilis* and *S. sonnei*. The results in this study have correlation with those obtained with methanol: acetone: diethyl ether extract, where all the studied algae gave inhibitory effect toward one of the targeted bacteria at minimum, with *T. distorta* having the most effective activity against *S. aureus*, *B. subtilis* and *S. sonnei*.

Meanwhile, acetone extract of *M. aeruginosa* recorded good activity (~20 mm) against *B. subtilis* and slight activity (~16 mm) against *S. aureus*, at the same time, *T. distorta* was found active only against the latter. These results are in agreement with that previously screened for these bacteria (Mian et al., 2003; Volk et al., 2006; Issa, 1999). *D. salina* gave a slight activity against *P. aeruginosa* contrary to the negative effect recorded by methanol: acetone: diethyl ether and ethanol extracts. Our extracts prepared with ethanol exhibited activity against *S. aureus* and *B. subtilis* for both *P. autumnale* and *T. distorta*. The mode of action of different extracts was proven to be similar within some algal species as reported by Patra et al. (2009), and noticed by comparing methanol: acetone: diethyl ether versus ethanol extracts of *P. autumnale* toward *B. subtilis*, as same as

Table 1. Antibacterial activity of different extracts of algae using three different solvent systems.

Algal species	Organic extractant	Gram negative bacteria				Gram positive bacteria		Yeast
		S. suis	***P. aeruginosa***	***E. coli***	***S. sonnei***	***S. aureus***	***B. subtilis***	***C. albicans***
C. vulgaris	Acetone/methanol/diethyl ether	_	_	_	+++ (~20 mm)	_	++ (`18 mm)	_
	Acetone	_	_	_	_	_	_	_
	Ethanol	_	_	_	_	_	_	_
S. platensis	Acetone/methanol/diethyl ether	_	_	_	+++ (~22 mm)	_	_	_
	Acetone	_	_	_	_	_	_	_
	Ethanol	_	_	_	_	_	_	_
N. linckia	Acetone/methanol/diethyl ether	_	_	_	+++ (`~22 mm)	+	_	_
	Acetone	_	_	_	_	_	_	_
	Ethanol	_	_	_	_	_	_	_
P. autumnale	Acetone/methanol/diethyl ether	_	_	_	+++ (~25 mm)	_	++	_
	Acetone	_	_	_	_	_	_	_
	Ethanol	_	_	_	_	+++ (~20 mm)	+++ (~21 mm)	++ (~18 mm)
D. salina	Acetone/methanol/diethyl ether	_	_	_	++	_	_	_
	Acetone	_	+(~8.5 mm)	_	_	_	_	_
	Ethanol	_	_	_	_	_	++	_
T. distorta	Acetone/methanol/diethyl ether	_	_	_	+++ (~25 mm)	++ (~19mm)	+++ (~25 mm)	_
	Acetone	_	_	_	_	_	+	_
	Ethanol	_	_	_	_	+ (~9.0 mm)	+ (~9.0 mm)	_
M. aeruginosa	Acetone/methanol/diethyl ether	_	_	_	+ (~10 mm)	+	_	_
	Acetone	_	_	_	_	++ (~16 mm)	+++ (~20 mm)	_
	Ethanol	_	_	_	_	_	_	_

(-) No activity; (+) weak activity (~8.0 – 15 mm); (++) moderate activity (~16 mm-19 mm); (+++) high activity (~20-25 mm).

that of *T. distorta* toward *S. aureus* and *B. subtilis*.

However, *D. salina* was found active only against *B. subtilis*, and also, *C. albicans* was proven to be inhibited only by *P. autumnale* among the other extracts of the same test species. Many investigations addressing ethanol extracts of various algal species have revealed activity against Candida species (Abedin et al., 2008; Tüney et al., 2006), which is in good agreement with our finding.

In accordance to our results, some of the tested algae exhibited good bioactivity upon extraction with specific solvent, confirming that there is multi-factorial intra-specificity of the active metabolites in the studied species, which was affected by solvent selection and extraction process (Perez et al., 1990; Gonzalez et al., 2001).

As the strains selected in the present study showed good antibacterial activity, further studies regarding the extraction and purification of antibacterial metabolites are in progress. It is

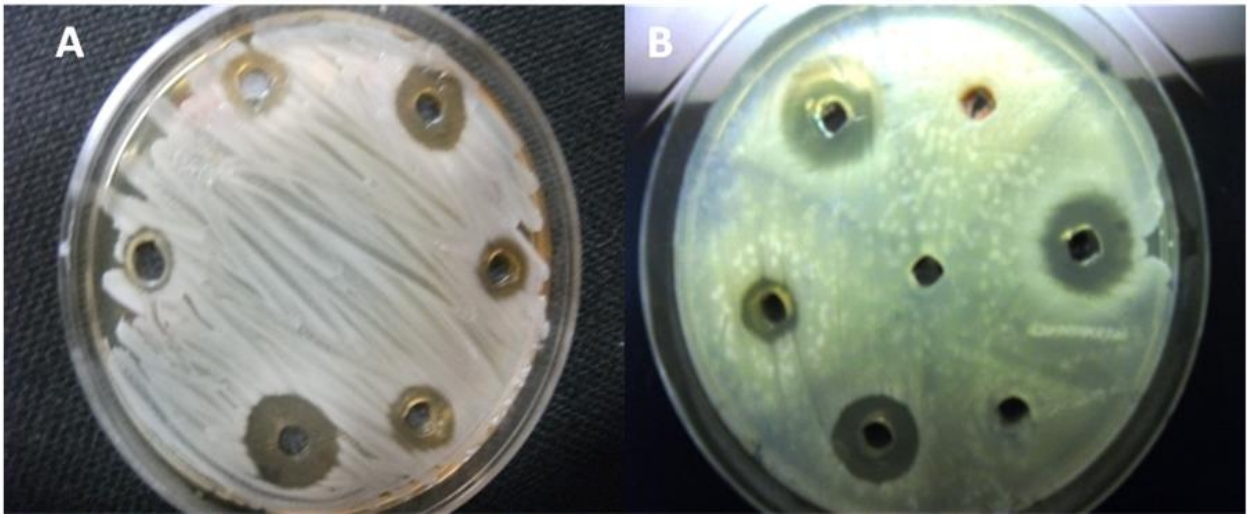

Figure 3. Antibacterial activity of methanol: acetone: diethyl ether extracts of *C. vulgaris*, *P. autumnale* and *T. distorta* against *S. sonnei* (A), and *B. subtilis* (B).

hoped that the seven strains may provide the most promising array of pharmacologically new antibacterial compounds which will improve our armamentarium for prevention and treatment of life threatening bacterial infections in human life.

This study was only based on crude extracts and did not indicate any defined antimicrobial substance. Thus, further investigations have to be made on fractionation, in order to identify the principal bioactive compounds in addition to the factors affecting their mode of action.

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