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Phytochemical screening and antimicrobial activities of some green algae from Egypt

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Abstract
Chlorophytes represent the largest group as eukaryotic photosynthetic green organisms. Most micro- and macro-green algae are potential sources for active compounds. *Ulva sp, Enteromorpha sp,* and *Chlorella sorokiniana* were isolated from the Mediterranean Sea, Port Said, Egypt. The phytochemical constituents (alkaloids, saponins, tannins, terpenoids, flavonoids, cardiac glycosides and anthraquinone) and the antimicrobial properties were investigated for all species` extracts. Cells were dried then grounded. The powdered biomass was soaked into different solvents (methanol and acetone). The antimicrobial activities of different fraction were done using the disc diffusion technique. Results indicated the presence of different active compounds, and the antimicrobial property in all species which increase the importance of them. The phytochemical screening of methanolic and acetonic extract revealed the presence of phytoconstituents including anthraquinones, cardiac glycosides, flavonoids, saponins, tannins and terpenoids. The antimicrobial assessment confirmed the effect of *C. sorokiniana* extracts on the growth of *Escherichia coli* and *Staphylococcus aureus*.

Keywords: Phytochemical constituents, antimicrobial property, *Ulva sp*, *Enteromorpha sp*, *Chlorella sorokiniana*

1. Introduction
Recently, it was investigated that the aquatic habitats are potential feedstocks of natural bioactive compounds with hypocholesterolemia, anti-inflammatory, antiviral, antineoplastic, antimicrobial and hypertensive properties especially, sulfated polysaccharides, phenolics, terpenoids, lactons, sterol and fatty acids [1, 2, 3]. Natural bioactive compounds are compounds which extracted from living organisms such as, algae, plants, and animal sources [4]. These compounds are classified into terpenoids, polyketides, amino acids, peptides, proteins, carbohydrates, lipids and nucleic acid bases [5].

Chlorophytes represent the largest group as eukaryotic photosynthetic green organisms that have chlorophyll a and b. Most micro- and macro-green algae are potential sources for active compounds. Significant amounts of essential proteins, vitamins, and minerals were found in different chlorophytes [6]. Green seaweeds (macroalgae) had different antioxidants, antibiotics, and bioactive compounds as carotenoids, phenolics, terpenoids and sulphated polysaccharides exhibit potent antioxidant activities [7].

The sea-lettuce; *Ulva* sp. is one of the most common and abundant green macro algae throughout the world. However, its wide distributed and has many nutritional components like, vitamins, oligo-elements, minerals, and dietary fibers, it is poorly utilized [8].

Enteromorpha is a marine chlorophyte which is widespread in the tropical waters. It has recently been reported that scientists have extracted a potential anti-Tuberculosis drug from *Enteromorpha* that showed antibacterial activity against nearly twenty disease causing-microorganisms [9].

Chlorella sorokiniana (known previously as *Chlorella pyrenoidosa*) is a unicellular alga with identified C-shaped chloroplast. It has a high chlorophyll content, nutritional components, antioxidants, and antibiotics. It has a natural antibiotic called “Chlorellin” that combats pathogenic bacteria without altering the intestinal flora [10]. C. *sorokiniana* had many applications as bioremediation agent, biofertilizer, and energy feedstock.

Phytochemical studies have attracted the attention of researchers due to the development of new and sophisticated techniques, and to enhance the resistance and immune system against different pathogens.

This study aims to screen the Phytochemical and microbial activities of *Ulva sp, Enteromorpha sp,* and *Chlorella sorokiniana* to reveal its secondary metabolite constituents for various medical and industrial application.

2. Material and methods

2.1 Sources of algal strains

Chlorella sorokiniana samples obtained from Culture Collection Unit at phycological lab in Faculty of Science, Port Said University, Port Said, Egypt. Whereas, seaweeds (*Ulva* and *Enteromorpha*) were handpicking from the rocky substratum along the subtidal areas at the Mediterranean coast, Port Said, Egypt (Figure 1).

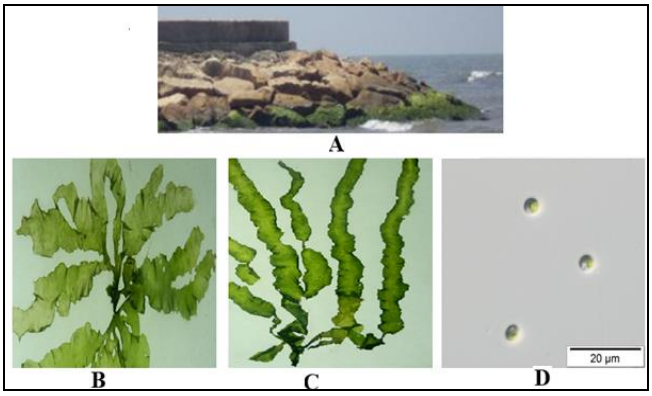

Fig 1: The rocky substratum along the subtidal areas at the Mediterranean coast, Port Said, Egypt (A). Other images showed algal strains as follow: *Ulva* sp. (B), *Enteromorpha* sp. (C), and *Chlorella sorokiniana* (D)

2.2 Preparation of samples

2.2.1 Microalgae

Chlorella sorokiniana refreshed by adding 300 ml sterile BG-11 medium to 200 ml of the pure culture in 1L sterilized flask and gently vortexed daily. After 10 days, 500 ml sterile BG-11 medium added to an equal volume of the maintained culture and gently mixed. The algae were incubated at room temperature of 27 °C±1 with continuous lighting of 80 $\mu molm^{-2}sec^{-1}$. After certain period, the culture was left to settle for 2 hours then the precipitated biomass was harvested. This biomass was placed inside 35 °C oven for 2 days to be gently dried.

BG-11 medium composed of (g l^{-1}), $NaNO_3$, 1.5; $K_2HPO_4 \cdot 3 H_2O$, 0.04; $MgSO_4 \cdot 7 H_2O$, 0.075; $CaCl_2 \cdot 2 H_2O$, 0.036; citric acid, 0.006, ferric ammonium citrate, 0.006; Na_2EDTA, 0.001; Na_2CO_3, 0.02. In addition to 1 ml of trace metal solution (including H_3BO_3, 2.86 g; $MnCl_2 \cdot 4 H_2O$, 1.81 g; $ZnSO_4 \cdot 7 H_2O$, 0.222 g; $Na_2MoO_4 \cdot 2 H_2O$, 0.390 g; $CuSO_4 \cdot 5 H_2O$, 79 mg and Co $(NO_3)_2 \cdot 6 H_2O$, 49.4 mg l^{-1}) [11].

2.2.2 Macroalgae

Collected masses of *Ulva* and *Enteromorpha* were cleaned and washed with tab water to remove dust. Their fresh weight was measured using a Sartorius Entris® Balance (224-S1, Germany). The seaweeds were air dried at room temperature for 7 days. Then grind mechanically.

2.3 Preparation of extracts

Different solvents (Methanol and Acetone) were used to prepare the extracts from the micro- and macro- powdered biomass using sequential extraction. Each of the pooled extract was concentrated under vacuum at low temperature to get the crude extracts from each solvent used and reflux method.

2.3.1 Extraction with Methanol

Solid-liquid extraction process were used to separate soluble components from a solid sample three times. A definite dry weight for each alga was placed inside the thimble. Fifty ml of methanol was separately placed in the still pot. The extraction Soxhlet (Figure 2) operated for 3 hours. The methanol fraction gained after the extraction were concentrated using the rotary evaporator (RE300, England) under reduced pressure at 45 °C reflux method [12]. The remains biomass was discarded at the end of the extraction process.

Fig 2: Extraction set up.

2.3.2 Extraction with Acetone

Solid-liquid extraction process were used to separate soluble components from a solid sample three times. A definite dry weight for each alga was placed inside the thimble. Fifty ml of acetone was separately placed in the still pot. The extraction Soxhlet operated for 3 hours. The acetone fraction gained after the extraction were concentrated using the rotary evaporator (RE300, England) under reduced pressure at 45 °C reflux method [12]. The remains biomass was discarded at the end of the extraction process.

2.4 Phytochemical screening

Suitable amounts of samples were extracted using absolute methanol and acetone. The extracts were evaporated to incipient dryness respectively and tested for the presence of secondary metabolites e.g., Alkaloids, Anthraquinones, Cardiac Glycosides, Flavonoids, Saponins, Tannins, and terpenoids according to the common chemical methods described by [13].

2.4.1 Test for Alkaloids

Solvent free extract was stirred with 1.5 ml HCl (2%) then filtered. The filtrate was tested carefully with adding 1 to 2 drops of Mayer's reagent. A white-creamy precipitate indicated the presence of the alkaloidal base [14].

2.4.2 Test for Anthraquinones

According to the Borntrager's Test [15], 5 ml of the extract was added to 5 mL of chloroform was added and shake for 5 min then filtered. Two ml of the filtrate was added to 1ml of dilute 10 % ammonia solution and shake. A pink violet color in the Ammoniacal layer (lower layer) indicates the presence of Anthraquinone.

2.4.3 Test for Cardiac Glycosides

According to the Keller Killiani Test [16], 5 ml of the extract was tested by adding 1 mL of glacial acetic acid containing one drop of ferric chloride solution under layered with 1 mL of concentrated Sulfuric Acid. A brown ring obtained at the

interface layer was indicated the presence of a de-oxy sugar characteristic of Cardenolides.

2.4.4 Test for Saponins
Five ml of distilled water added to 5 ml of the extract and shake vigorously. The formation of a stable persistent froth indicated the presence of Saponins [16].

2.4.5 Test for Tannins
Ten ml of the extract was boiled within 5 ml of water in a test tube then filtered. A few drops of 0.1% ferric chloride was added and either a brownish green or a blue-black color were observed [15].

2.4.6 Test for Terpenoids
According to Salkowski Test [17], Two ml of chloroform added to 5 ml of the extract and 3 ml of concentrated Sulfuric acid. A reddish-brown coloration of the interface layer indicated the presence of Terpenoids.

2.4.7 Test for Flavonoids [18, 19]
Ammonium/Sulfuric acid test– 5 ml of dilute ammonia, and 1 ml of concentrated Sulfuric acid were added to the extract, respectively. The disappearance of the yellow color indicated the presence of Flavonoids.
Ammonium/Sulfuric acid test– Formation of yellow color as a result of adding few drops of 1% aluminum solution were added to the extract indicated the presence of flavonoids.
Acetate acid test– a portion of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. Then the mixture was filtered, and 4 ml of the filtrate shaken with 1 ml of dilute ammonia solution. The yellow color indicated the presence of flavonoids.

2.5 Microbial pathogens
Three bacterial pathogens were used to indicate the antimicrobial activity of *C. sorokiniana*. *Escerichia coli*, *Staphylococcus aureus* and *Streptococcus* sp. were obtained from the Port Said medical hospital, Port Said, Egypt.

2.6 Antimicrobial susceptibility test
Disc diffusion method [20] was performed to show the effect of different extracts on growth of three bacterial strains; *Escerichia coli*, *Staphylococcus aureus* and *Streptococcus* sp. Sterilized Whatman No. I filter papers with 5-mm diameter were fattened with the different crude extracts. On the surface of a sterilized solid Nutrient Agar, the bacteria strains were streaked and left to grow for 24 h prior to use. On the surface of new sterilized solid Nutrient Agar, the bacteria were streaked, and the impregnated filter paper disks were placed on medium surface then the plates were incubated at 27 °C for 24 hours. The diameter of the inhibition zones was measured. The antibacterial assay was carried out in triplicate. Positive and negative control were applied. Chloramphenicol (500 mg/ml) was used as a positive control where the solvents were used as a negative control.

3. Results and Discussion
3.1 Phytochemical screening
In this study, the micro- and macro-algae *Ulva* sp, *Enteromorpha* sp, and *Chlorella sorokiniana* were selected and screened for the presence of phytochemical constituents. The phytochemical constituents such as alkaloids, saponins, tannins, terpenoids, flavonoids, cardiac glycosides and anthraquinone that serve as defense mechanism against different infectious disease were screened.
The phytochemical screening of methanolic and acetonic extract revealed the presence of phytoconstituents including anthraquinones, cardiac glycosides, flavonoids, saponins, tannins and terpenoids. Methanol was found to be the most prominent solvent in extracting the major phytoconstituents from the studied algal species biomass compared to acetone. The alkaloids were obviously absent in the extracts of all tested genera which was not consistent with [21] where alkaloids were found in *Ulva*.
The presented results indicated that *Ulva* had Anthraquinones, cardiac glycosides, saponins, tannins and terpenoids. While, *Enteromorpha* had flavonoids instead of Anthraquinones in *Ulva*. Whereas, *C. sorokiniana* had all bioactive compounds which found in *Ulva* and *Enteromorpha* (Table 1).

Table 1: Phytochemical screening on the methanolic extracts for *Ulva* sp., *Enteromorpha* sp. and *Chlorella sorokiniana*

Bioactive compounds	*Ulva* sp.	*Enteromorpha* sp.	*Chlorella sorokiniana*
alkaloids	+	–	+
saponins	+	+	+
Tannins	+	+	+
terpenoids	+	+	+
flavonoids	–	+	+
cardiac glycosides	+	+	+
anthraquinone	+	+	+

* + means Presence of constituents; whereas – means Absence of constituents.

The results of study conceded with the earlier studies on chlorophycean members *Desmococcus olivaceous*, *Chlorococcum humicola* and *Chlorella vulgaris* which showed the presence of cardiac glycosides, terpenoids and saponins [22, 23].
Flavanoids are compounds that can be used as an antioxidant, anti-allergic, anti-inflammatory, anti-microbial, and anti-cancer agent. The presence of flavanoids in *Enteromorpha* sp. and *Chlorella sorokiniana* reveal these species as potential sources for different medicines [24, 25].
Phenolic compounds such as flavonoids, tannins, and simple phenols are critical defenses compounds against environmental stress and pathogenic bacteria [13]. Tannins has recently been found as antiviral, antibacterial, and antiparasitic agents [28]. Tannins can inhibit enzymes activity [26, 27]. The existing phenolic compounds inside tested organisms confirmed their antimicrobial activity.

3.2 Antimicrobial activity of *C. sorokiniana*
The growth of *Escherichia coli* and *Staphylococcus aureus* was negatively affected by acetone and methanol extracts of *C. sorokiniana*, correspondingly. A clear zone around the discs in *Escherichia coli* and *Staphylococcus aureus* plates was observed (Figures 3, 4). These results were agreed with the earlier reports that the methanol extract of *Desmococcus olivaceous*, *Chlorococcum humicola*, and *Chlorella vulgaris* were effective in exhibiting antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* [22, 23]. While

Streptococcus growth was not affected by neither methanol nor acetone extracts (Figure 5). The clear zones of the discs with extracts have small diameters compared to antibiotic disc (positive control), this is may be due to the low concentration of phytoconstituents in extracts than in used antibiotic. In this context the active metabolites present in extracts of the *C. sorokiniana*, may be associated with their antimicrobial activity.

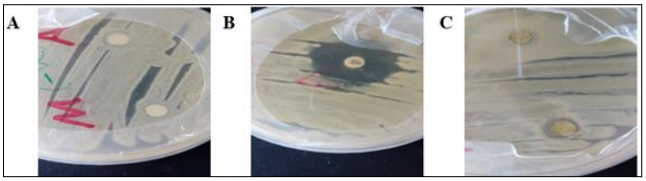

Fig 3: Effect of *Chlorella sorokiniana* extracts on the growth of *Staphylococcus aureus* using disc diffusion method. Where, A; negative control, B; positive control, and C; the tested extracts.

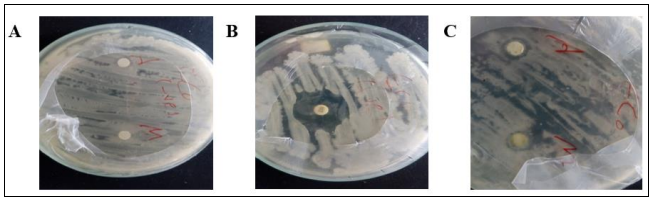

Fig 4: Effect of *Chlorella sorokiniana* extracts on the growth of *Escherichia coli* using disc diffusion method. Where, A; negative control, B; positive control, and C; the tested extracts.

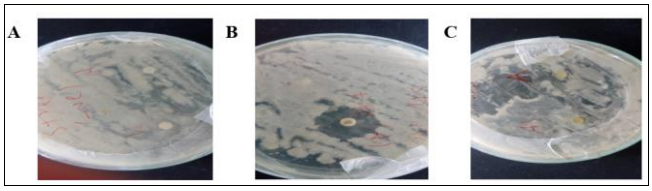

Fig 5: Effect of *Chlorella sorokiniana* extracts on the growth of *Streptococcus* sp. using disc diffusion method. Where, A; negative control, B; positive control, and C; the tested extracts.

Different scientists had studied the antibacterial activities of different algae strains against different pathogens [29]. Different factors affect the antimicrobial activity as sample collection (period and place), and the extraction and the assessment protocols [30, 31]. Many solvents were tested, methanol and ethanol extracts were active only against *Shigella flexneri* [32].

4. Conclusion

In present study, we demonstrated the feasibility for This research about phytochemistry of algae, in which three chlorophytes species (*Ulva* sp. and *Enteromorpha* sp. and *C. sorokiniana*) were studied for their phytoconstituents. These phytoconstituents are used in the production of many drugs, cosmetics and in pharmaceutical industries. For extraction of phytoconstituents, the serial exhaustive extraction method was used. The phytochemical screening of methanolic and acetonic extract revealed the presence of phytoconstituents including anthraquinones, cardiac glycosides, flavonoids, saponins, tannins and terpenoids. The extracts of *C. sorokiniana* were tested as antimicrobial activity against three bacterial strains (*Escherichia coli*, *Staphylococcus aureus* and *Streptococcus* sp.) using the disc diffusion method. The results show sensitivity of *Escherichia coli* and *Staphylococcus aureus* to both methanol and acetone extracts while *Streptococcus* sp. was resistant to both extracts. These promising findings may be a potential to utilize such extracts as antimicrobial agent and as natural immunostimulant for aquaculture.

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