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Production and characterization of antifungal active substance from some marine and freshwater algae

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ABSTRACT

The biological activity of two freshwater algae species; *Spirulina platensis* and *Chlorella vulgaris* and two seaweeds; *Sargassum vulgaris* and *Sargassum wightii*, was tested *in vitro* against *Trichophyton rubrum*, *Microsporum canis* and *Candida albicans*. The results showed that all algal extracts exhibited inhibitory activity against the studied fungi. Seventy percent methanol extracts from *Chlorella vulgaris* showed the highest inhibition against the tested microorganisms. The results of this investigation suggested that the methanol extract from *Chlorella vulgaris* contains a new antifungal compounds. The purification and determination of the chemical structure of active compounds were investigated.

1. INTRODUCTION

Fungi as agents of superficial mycoses cause a wide range of diseases in humans and animals, involving the outer layer in the stratum corneal of the skin and frequently causing chronic infections. The major etiological agents of these mycoses are dermatophytes and *Candida* species, cosmopolitan fungi that are able to affect deeper layers of the epidermis and mucous or organs in debilitated individuals. As the population of immune-compromised continues to arise, the opportunistic fungal pathogens infecting these patients continue to increase as well (Hay and Jones, 2005).

Microalgae are currently cultivated commercially for human nutritional products around the world in several dozen small- to medium-scale production systems; each producing a few tens to several hundred tons of biomass annually. Benemann (2009) estimated total world commercial microalgal biomass production at about 10,000 tons per year. The main algae currently cultivated photosynthetically for nutritional products are *Spirulina, Chlorella, Dunaliella* and *Haematococcus*. About half of the microalgae production takes place in mainland China, with most of the rest in Japan, Taiwan, USA, India and Australia, with smaller producers in other countries. Almost all (~99%) commercial production is carried out with open ponds. It is known to produce intracellular and extracellular metabolites with diverse biological activities such as antifungal (MacMillan *et al*., 2002), antiviral (Hayashi and Hayashi, 1996), and antibacterial activities (Kaushik and Chauhan, 2008).

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The ability of seaweeds to produce secondary metabolites of potential interest has been extensively documented (Faulkner, 1993).

There are numerous reports of compounds derived from macroalgae with a broad range of biological activities, such as antibiotics (antibacterial and antifungal properties), antiviral diseases (Trono, 1999), antitumor and anti-inflammatory (Scheuer, 1990) as well as neurotoxins (Kobashi, 1989). Chemical structure types include sterols (Ahmad *et al*., 1993), isoprenoids amino acids, terpenoids, phlorotannins, steroids, phenolic compounds, halogenated ketones and alkanes, cyclic polysulphides, fatty acids and acrylic acid can be counted (Mtolera and Semesi, 1996).

The ability of seaweeds to produce secondary metabolites of antimicrobial value, such as volatile components (phenols and terpenes) (Cox *et al*., 2010; Gupta and Abu-Ghannam, 2011), steroids (Shanmughapriya *et al*., 2008), phlorotannins (Wang *et al*., 2009) and lipids (Shanmughapriya *et al*., 2008) has been already studied. Among these, phlorotannins as polyphenolic secondary metabolites are found only in brown algae (Heo and Jeon, 2005).

This work aims to evaluate the antifungal activity of two species of seaweeds and two species of fresh microalgae with various extraction solvents against *Trichophyton rubrum, Microsporum cani s*and *Candida albicans* in order to discover new natural antifungal compound.

2. MATERIALS AND METHODS

2.1 Microalgae

Two algal strains *Spirulin aplatensis* and *Chlorella vulgaris* were obtained from the culture collection of the Botany Department, Faculty of Science, Mansoura University, Egypt. Zarrouk's (1966) and Kuhl's (1962) media were used for cultivation of *S. platensis* and *C. vulgaris*. The culture flasks were aerated with air mixed with 3% CO_2 to accelerate algal growth and incubated at 25ºC for *Chorella* and 30ºC for *Spirulina*, under continuous illumination provided from day light fluorescent tubes giving light intensity of 80 $\mu Em^{-2}s^{-1}$ for *C. vulgaris* and 46$\mu Em^{-2}s^{-1}$ for *S. platensis*.

2.2 Macroalgae (seaweeds)

The two species seaweeds *Sargassum vulgare* and *Sargassum wightii* (Phaeophyta) were collected from the rocky areas in few meters below the water in Red sea, Seuz beach, Egypt during November 2012. The seaweeds were brought to the laboratory in plastic bags containing sea water to prevent evaporation. Algae were then cleaned from epiphytes and rock debris and given a quick fresh water rinse to remove surface salts. The seaweeds were identified following Abbott and Hollenberg (1976), Aleem (1993) and Taylor (1985). The samples were air dried in the shade at room temperature 25 °C: 30 ºC on absorbent paper, cut into small pieces and grounded to fine powder.

2.3 Algal extracts

In case of microalgae (*S. platensis* and *C. vulgaris*) the algal powder was soaked for 5h at room temperature and sonicated for 15 minutes, while the extracts of macroalgae were kept in the respective solvent for a certain period on a rotator shaker at 150 rpm at room temperature (25°C: 30°C) and the resultant crude extracts were filtered through Whattman filter paper no. (1), the obtained filtrate was freed from solvent by evaporation under reduced pressure. The obtained residues (crude extracts) were suspended in the respective solvents to final concentration of 50 mg/ml. The extraction with different solvents was carried out individually on samples. The extract was stored at -20 ºC in airtight glass bottle for the antimicrobial assay.

2.4 Fungal isolates

The fungal species were collected from different patients clinically diagnosed to have different locations and shapes of

tineacorporis and cutaneous candidacies lesions. This was achieved by visiting the outpatient clinic of Dermatology Department at Tanta University Hospital daily along 3 months from June to August 2013. The best purified isolates were used in our study. Each sample was cultured in three sterilized petri dishes containing sterile Sabouraud's dextrose agar (SDA) medium; each 1 liter of distilled water contained 30 gm dextrose, 10 gm peptone, 20 gm agar, and 0.5 g from each chloramphenicol and cycloheximide were added to avoid bacterial contamination (Al Doory, 1980). Plates were incubated at 37 °C for 7-20 days for dermatophytes and for 2-3 days for *Candida albicans*.

All cultured plates were examined under microscope (400X), identified and photographed. Infected lesions on skin were photographed and clinically described, from each patient all information were recorded included personal data (age, sex and no. of family), climate, occupation, medical history, location and site of infection. Also the scales of patient examined under microscope (400X 100X) and photographed. The tested organisms were inoculated on nutrient agar medium and incubated for 24 h at 37°C for bacteria and on sabouraud agar media for 72h and 15 days at 25 °C for yeast and the dermatophytes, respectively using slope culture, then they were transferred directly to a refrigerator at +4 °C and were subcultured every two months on the same media.

2.5 Determination of the best solvent for extracting the antifungal material from micro and macroalgae

Cut plug method described by Pridham, *et al.* (1956) was employed to determine the antifungal activity of each algal extracts. Three replicates were made for each test suspension and the plates of dermatophytes and yeast were incubated at 37 °C for 15 days and 3 days, respectively. Then the average diameters of inhibition zones were recorded.

2.6 Determining the minimum inhibitory concentration (MIC) for fungi

MIC was determined by agar method (Chattopadhyay *et al*., 1998). Serial dilutions (200, 100, 50, 25, 12.5, 6.25, 3, 1 mg/ml) of the antimicrobial material were tested against three pathogenic fungi with concentration (10^8 CFU) namely (*Trichophyton rubrum, Microsporum canis and candida albicans*). The lowest concentration which did not show any visible growth was considered as the MIC.

2.7 Statistical analysis

Results are presented as mean ± SD (standard deviation) for three replicates. The statistical analysis were carried out using SAS and SYSTAT statistical software packages, the degree of significance of the obtained results was tested. One and three way analyses of variance were carried out.

3. RESULTS AND DISCUSSION

The results presented in Table 1 showed that ethanol, methanol, ethyl acetate and chloroform extracts of different algae (*Spirulina platensis, Chlorella vulgaris, Sargassum vulgare* and *Sargassum wightii*) possessed antifungal activity against the microorganisms tested. Inhibition activity percentage = (average diameter of inhibition zone of each solvent/ average diameter of inhibition zone of all solvent s) x100. As obtained from results methanol extracts have the strongest inhibition against the tested microorganisms with inhibition activity percentage of 42.3%, followed by ethanol extracts with inhibition activity percentage of 33.5%. However ethyl acetate extractshave 14% inhibition activity, whereas chloroform showed the lowest inhibition activity percentage of 10.3% for all algae extracts against all tested fungi. Therefore, methanol was the best solvent for extractions. As observed from data, *Chlorella vulgaris* gave the highest inhibition zone among the other algae, followed by *Spirulina platensis, Sargassum wightii* and *Sargassum vulgare*. One-way analysis of variance (ANOVA) confirmed that the variation in antifungal activities in relation to algae and fungi were

significant at P≤ 0.001 for almost treatments except *S. platensis*, *S. vulgare*, *S. Wightii*a against *Trichophyton rubrum* and *S. wightii* against *Microsporum canis* were significant at P ≤0.01. On the other hand, *S. platensis* against *Candida albicans* and *C. vulgaris* against *Microsporum canis* were non-significant at P≥0.01 (Table.1).

Table 1.One-way analysis of variance (ANOVA) showing the antifungal activity of different algae extracted with different solvents measured by (mm).

Algae	Solvent (70%)	Fungi		
		Trichophyton rubrum	***Microsporum canis***	***Candida albicans***
Spirulina platensis	Ethanol	23.67±1.16	25.00±0.0	29.33±1.16
	Methanol	27.67±0.58	28.67±1.16	35.67±1.16
	Ethyl acetate	9.33±1.16	9.33±1.16	19.33±1.16
	Chloroform	9.67±0.58	9.33±1.16	10.67±1.16
F-Value		4.33*	2.17**	1.33^{ns}
Chlorella vulgaris	Ethanol	28.67±1.16	34.33±1.16	38.33±1.53
	Methanol	38.67±1.53	40.33±0.58	45.33±1.53
	Ethyl acetate	18.33±1.53	22.00±1.73	28.00±0.0
	Chloroform	12.00±0.0	14.67±0.58	16.33±1.16
F-Value		3.00**	1.33^{ns}	3.00**
Sargassum vulgare	Ethanol	20.33±0.58	18.67±1.53	20.67±0.58
	Methanol	23.33±1.16	19.33±1.16	30.67±1.16
	Ethyl acetate	0.00±0.0	0.00±0.0	10.00±0.0
	Chloroform	0.00±0.0	5.33±0.58	6.33±1.16
F-Value		7.17*	3.17**	2.00**
Sargassum wightii	Ethanol	21.00±0.0	20.33±1.53	23.00±0.0
	Methanol	25.67±0.58	28.67±1.16	39.33±1.16
	Ethyl acetate	0.00±0.0	0.00±0.0	10.67±0.58
	Chloroform	0.00±0.0	0.00±0.0	8.67±1.53
F-Value		4.67*	10.00*	2.00**

(±) standard deviation of the means (n=3); *Significant at $P \leq 0.01$, **Significant at $P \leq 0.001$, and (^{ns}) Non-significant at $P \geq 0.01$.

As shown in Figure 1, methanol extract of *Chlorella vulgaris* is the most effective one against dermatophytes and yeast. On the other hand, the chloroform extract showed a weak effect on dermatophytes (*Trichophyton rubrum* and *Microsporum canis*) and yeast (*Candida albicans*).

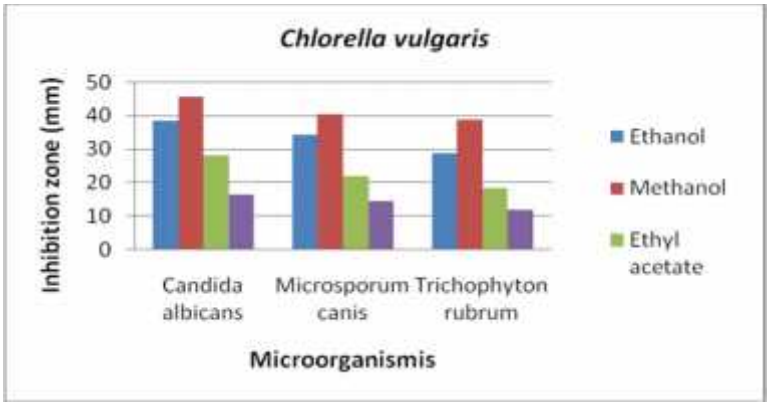

Fig. 1: Different extracts of *Chlorella vulgaris* against dermatophytes and yeast.

Figure 2 shows that methanol extract of *Spirulina platensis* gave a high inhibitory effect against yeast, and then dermatophytes, followed by ethanol extract. However, the chloroform and ethyl acetate extracts showed a weak effect on dermatophytes and yeast.

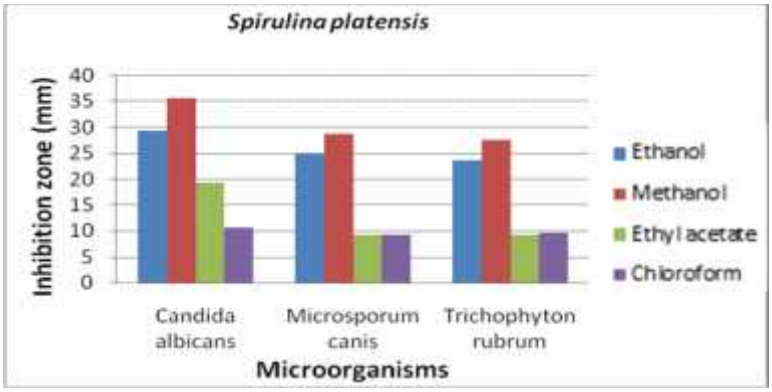

Fig. 2: Different extracts of *Spirulina platensis* against dermatophytes and yeast.

Methanol extract of *Sargassum vulgare* gave a high inhibitory effect against dermatophytes and yeast. On the other hand, ethyl acetate and chloroform extracts have no antifungal activity against dermatophytes but gave a weak effect against *Candida albicans* (Figure 3).

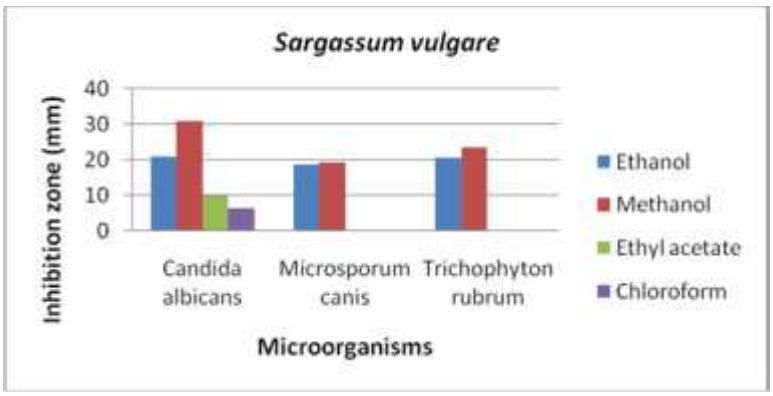

Fig. 3: Different extracts of *Sargassum vulgare* against dermatophytes and yeast.

In the present study, Figure 4 showed that methanol extract of *Sargassum wightii* gave a high inhibitory effect against dermatophytes and yeast, followed by the ethanol extract. On the other hand, the ethyl acetate extract has no antifungal activity against *Trichophyton rubrum* and *Microsporum canis* and a weak effect against *Candida albicans*, but the chloroform extract gives a weak effect against *Microsporum canis* and *Candida albicans*and no antifungal activity against *Trichophyton rubrum*.

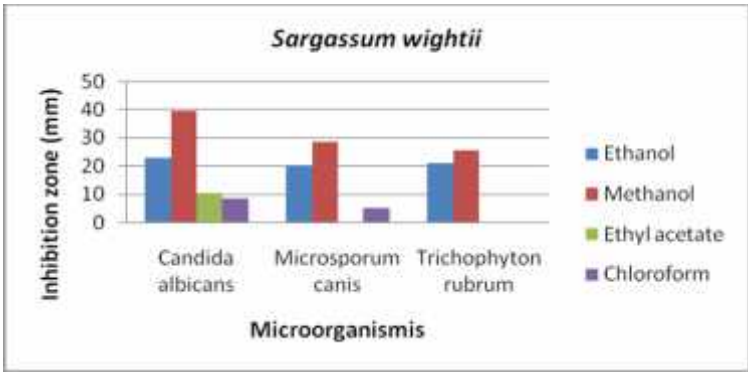

Fig. 4: Different extracts of *Sargassum wightii* against dermatophytes and yeast.

The organic solvent always provides a high efficiency in extracting compounds for antimicrobial activities compared to water-based methods (Axelsson and Gentili, 2014). Lipid-soluble extracts from fresh water microalgae and marine macroalgae have been investigated as a source of substances with pharmacological properties. Sudek (2006) recorded the antifungal activity against *Candida albicans* and antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecalis* of methanol extract of *Trichodesmium erythraeum* (Cyanobacteria). De Cano *et al.* (1990) reported that the methanol extract of *Nostoc muscorum* showed the highest antifungal activities against *Candida albicans.* Pandian *et al.* (2011) also tested petroleum-ether, chloroform and methanol of *Acanthaphora spiciferain vitro* for its antifungal activity against *Candida albicans, Microsporum gypseum, Aspergillus niger*, respectively by disc diffusion techniques. The methanolic extract of *Acanthaphora spicifera* showed higher antibacterial and antifungal activity compared to the other two extracts.

The results presented in Table 2 showed the MIC of the different algal extracts against the different isolates of fungi. The MIC of *Trichophyton rubrum* and *Microsporum canis* was 12.5 mg/ml; however, the MIC of *Candida albicans* was 3 mg/ml.

Table 2: Minimum inhibitory concentration (MIC) of the purified antimicrobial material extracted from *Chlorella vulgaris* by 70% methanol measured by (mm).

Microorganism	**Concentrations (mg/ml)**							
	200	100	50	25	12.5	6.25	3	1
Trichophyton rubrum	39.67 ±0.58	38.67 ±1.16	35.00 ±0.0	20.00 ±0.1	10.00 ±0.0	0.00 ±0.0	0.00 ±0.0	0.00 ±0.0
Microsporum canis	50.00 ±0.0	44.67 ±1.53	38.67 ±1.16	20.67 ±1.16	20.67 ±1.16	0.00 ±0.0	0.00 ±0.0	0.00 ±0.0
Candida albicans	50.33 ±1.53	48.67 ±1.16	44.00 ±0.0	30.33 ±1.53	30.00 ±0.0	19.33 ±1.16	14.67 ±0.58	0.00 ±0.0

Many *in vitro* methods have been used to detect the antimicrobial activity of algal material. The most commonly used methods are the agar disk/well diffusion assay and the agar/broth dilution assay (Patton *et al.*, 2006). Studies to date have used the popular agar disk/well diffusion method (Gutierrez *et al*., 2008), *Scytonema hofmannii* (Pignatello *et al*., 1983), *Hapalosiphon fontinalis* (Moore *et al*.,1987), *Anabaena* spp. (Frankmolle *et al*., 1992), *Microcystis aeruginosa* (Ishida *et al*., 1997), *Phormidium* sp. (Fish and Codd, 1994), have been reported as the main groups of microalgae producing antimicrobial substances.

4. CONCLUSION

Fresh water algae and seaweeds isolated from Egypt have been shown to possess a specific antifungal activity. The most effective extract was methanol extract from *Chlorella vulgaris*. These observations showed their importance as a potential source for biological active compounds such as antifungal substances.

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