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Assessment of Antagonistic Effect of Alcoholic Extract from Cyanophyta (*Spirulina Platensis*) Against Several Human and Plant Derived Pathogenic Fungi

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Abstract

Background: Blue-green algae have been proved to producing different bioactive compounds. For this reason *Spirulina platensis* isolates have been used in the present study as a biocontrol agent against several human and plant pathogenic fungi. Current study was aimed to investigate the use of crude extract of *Spirulina platensis* as antifungal agent by determining the antagonistic activity of methanolic extract of *Spirulina platensis* against several human and plant pathogenic fungi. Also, to determine the antifungal phytochemicals within algal alcoholic extract through GC-mass analysis. **Method**: Four different concentrations of alcoholic extract were prepared (100,75, 50 and 25mg/ml) from algal isolates and their antagonistic activity was investigated against molds isolated from spoiled fruits (two isolates of *Aspergillus niger*, *Aspergillus flavus*, *Mucor* sp. and *Botrytis* sp.) and against several *Candiada* spp. (*Candida albicans, Candida glurbrate, Candida fameta, Candida lustrans*) which were isolated from different clinical sources. **Results:** The results showed that algal extract displayed wide range of antagonistic activity against tested fungi depending on applied algal extract concentration and tested fungal species. Also, GC-Mass data analysis had been performed for algal extract and 36 different bioactive chemicals have been identified including eight compounds authenticated as having antifungal activity. **Conclusion**: *Spirulina* could be used as alternative drug to treat *Candida* sp. infections as well as added to food industries to enhance their nutritional value and simultaneously decreasing the possibility of food spoilage by molds.

Keywords*: algal extract, bioactivity, onychomycosis, fungi, candida spp.*

Introduction

Nature has been a source of therapeutic agents for thousands of years and a remarkable number of recent drugs have been isolated from environmental sources. In addition, biologically active constituents have recently received notable attention. Algae are rich sources of constituents that are novel in their structure and authenticated as biologically active metabolites, primary or secondary metabolic products produced by these organisms may exhibit potential bioactivity in pharmaceutical industry [(1)]. Blue-green algae have been proved to produce different bioactive compounds. These comprise antibiotics which inhibited microorganisms responsible for human and plants diseases[(2)].

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Spirulina is blue green algal genus belongs to Oscillatoriaceae which is characterized as free floating, spiral, multicellular and filamentous in appearance. The filaments are approximately 50-300µ long and 10µ in diameter and nitrogen-non fixing [(3)]. Fungi cause a broad range of superficial mycoses diseases in both humans and animals, involving the outer layers of the skin and frequently leadings to chronic infections. The major etiological agents of mycoses infections are *Candida* spp. and dermatophytes [(4)]. Also, fungal pathogens are important problem in agriculture, since most of the fungicides engaged exhibit lower effectiveness under field conditions and may cause chemical environmental pollution as well as poisoning of crops. In addition, some species, e.g. *Aspergillus flavus* can contaminate food and produce severe mycotoxins [(5)].

Spirulina as many other blue-green algae are producing varieties of antimicrobial agents, so they are

considered as suitable natural agents for manipulation as biocontrol mediators of plant pathogenic fungi and bacteria [1].

Therefore, current study was aimed to investigate the use of crude extract of *Spirulina platensis* as antifungal agent by determining the antagonistic activity of methanolic extract of *Spirulina platensis* against several human and plant pathogenic fungi. Also, to determine the antifungal phytochemicals within algal alcoholic extract through GC-mass analysis.

Materials and Method

Source of algae

Pre-isolated and characterized *Spirulina platensis* used in the present study was obtained from the laboratory of higher graduate at Biology Department in AL-Mustansiriyah University, Baghdad. The obtained isolate was cultured and maintained on BG-11 Medium and grown in an illuminated incubator.

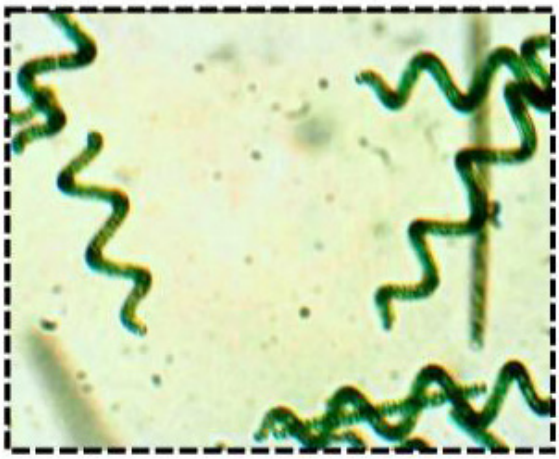

Figure (1) Microscopic view of *Spirulina platensis* (40X)

Preparation of algal Extract

Algal extract was prepared according to [6]. The *Spirulina platensis* materials were grounded to a fine powder and a weight of 40 gram from algal powder was extracted successively with 200ml of solvent (methanol) in Soxhlet extractor until the extract was clear. The extract was evaporated to dryness by reduced pressure using rotary vacuum evaporator and the resulting pasty form extracts were stored in a refrigerator at 4°C for future experiments.

Collection of human pathogenic tested Yeast

Four different species of *Candida* were obtained from the laboratory of higher graduate of Biology Department in AL-Mustansiriyah University, Baghdad. These species were (*Candida albicans, Candida glurbrate, Candida fameta* and *Candida lustrans*) . These yeasts were isolated from different clinical sources and maintained using Sabourauddextrose agar (SDA) medium according to [7].

Isolation and identification of tested plant pathogenic Molds

The examined plant-pathogenic molds used in this investigation were isolated from different local spoiled fruits according to [8] and were identified using cultural and morphological features such as conidial morphology, pigmentation and colony growth pattern, according to the technique of [9].

Bioactivity test of hot alcoholic extract of *Spirulina platensis* against molds

According to [10] technique that has been used for this test**,** different concentrations of hot alcoholic algal extract (100,75, 50 and 25mg/ml) were incorporated into PDA medium (potato dextrose agar) before pouring in Petri dishes. Also, clotrimazole (10µg/ml) was tested against tested molds to compare between the antifungal activity of natural agent (*Spirulina* extract) and industrial antifungal (clotrimazole). PDA medium Petri dishes without additions have been used as negative controls. All experiments ran in three replicates. Moreover, 3mm discs of fungal plugs were inoculated in the center of Petri dishes and incubated at 28±2°C for (8 – 10) days. The radial growth of the colony was measured. Percentage of inhibition of mycelial growth was calculated as follows:

%Radial growth Inhibition= [(R1- R2)/R1] x100

Where: R1is the average of radial growth in control plates; R2 is the average of radial growth in plates treated with algal extract or clotrimazole.

The spore suspension was collected from above culture. Through suspensions of spores in sterile DW then centrifuged. A hemocytometer was used to calculate the percentage of sporulation inhibition using the formula given by [11]:

Sporulation inhibition ratio= [(X-x) /X*100]

Where: X is the average number of spores in control plates; x is the average of spores' number in plates treated with nanoparticles.

Bioactivity test of alcoholic extract of *Spirulina platensis* against *Candida* spp

Antagonistic activity of algal extract was estimated in vitro by using well diffusion method according to [12]. Four concentrations of algal extracts had been tested (100,75,50 and 25mg/ml) and 100µL of each concentration was added to each well after spreading of each *Candida* sp. isolate by sterile loop and incubated at 37^0C for 24 hrs. Also, 10µg/ml clotrimazole was tested as positive control. All tests were performed in triplicate. Antifungal activity was determined by measuring the clear zone around the wells in millimeters.

Gas Chromatography-Mass Spectrometry

GC-Mass analysis was performed according to [13] using (SHIMADZU—Japan) and post run software. The phytochemical compounds have been identified by comparison of their mass with NIST library search and authentic standards.

Results and Discussion

Identification of isolated fungi

Five molds isolates were isolated from spoiled food and identified these molds were *Aspergillus niger* (two isolates), *Aspergillus flavus*, *Mucor* sp., *Botrytis* sp. These molds were used to estimate antifungal activity of *Spirulina platensis* crude extract. Some food-born fungi are implicated in human diseases. These fungal infections are categorized into systemic, subcutaneous, superficial and opportunistic [14]. Spores propagules from *Aspergillus and Mucor* pose dangerous public health problems. For instance, *A. niger* causes aspergillosis which is common amongst employees who inhale soil dust particles [14,15]. Inhalation of spores of *Mucor* spp. *and Aspergillus* spp. produces allergic reactions in human. These diseases are difficult to treat. Furthermore, *A. flavus* have been reported as dangerous to humans through causing aspergillosis that's associated high human mortality rates and producing serious toxins [16].

So that, there is a serious needs to improve naturally antagonistic products to eliminate food spoilage by these molds.

Bioactivity test of hot alcoholic extract of Spirulina platensis against molds

In current study, results of antagonistic activity of *Spirulina* methanolic extract revealed that algal extract displayed potential inhibition percentage against tested molds as compared to controls. Data from current study revealed that algal extract gave 89%, 78% and 95% inhibition in 100mg/ml against *Botrytis, Aspergillus flavus* and *Aspergillus niger* 2, respectively. In case of *Aspergillus niger* 1, there was 94% inhibition caused by algal extract in (75mg/ml), while 85% inhibition caused by fungicide (Figure 2). However, no growth inhibition was noticed against *Mucor* for both algal extract and clotrimazole.

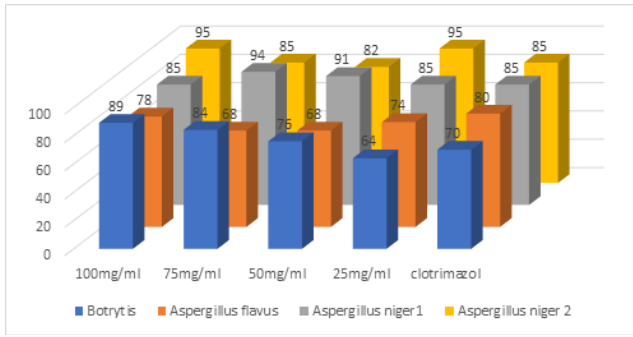

Figure (2) Growth inhibition percentages of different concentrations of *Spirulina platensis* methanolic extracts and clotrimazole against isolated plant-pathogenic molds.

As shown in Figure (3), spore germination was inhibited by *Spirulina* extracts. Spore germination of *Aspergillus flavus* was the most inhibited by algal extract (55%) at (100mg/ml), while *Botrytis* spore germination was less inhibited by algal extract (17.5%) at concentration (25mg/ml). Also, for *Botrytis* data, Figure (3) revealed that *Spirulina* extract (100mg/ml) inhibited spore germination for this mold by (44%). This percentage was higher than that caused by clotrimazole (28%).

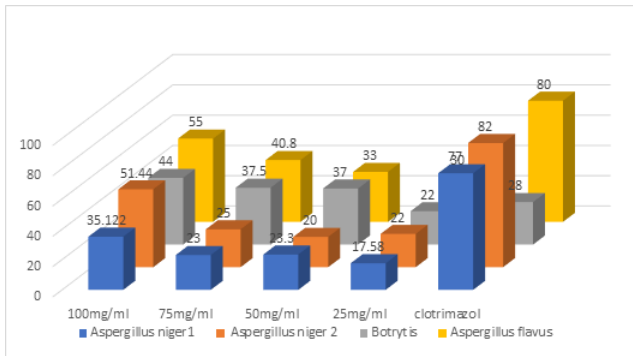

Figure (3) Spore germination inhibition percentages BY different concentrations of *Spirulina platensis* methanolic extracts and clotrimazole against isolated plant-pathogenic molds.

These findings agreed with other authors [1,2,17]. The previous authors tested *Spirulina* methanolic extracts against several fungi as *Candida* spp., *Aspergillus niger*, *Aspergillus flavus* and *Fusarium* spp. Their results showed that extract exhibited strong antifungal activity against these fungi. In addition, [2] reported that since the algal extracts are considered natural products which may have efficacy for fungal diseases management, a directed search is needed to understand culture conditions enhancing production of biologically active chemicals. During the present study, Spirulina have cultivated using BG-11 medium. The results of [2] indicated that *Spirulina* exhibited the highest antimicrobial effects when being cultured in BG-11 medium against tested microbes. For *Mucor* spp., algal extracts did not exhibit antagonistic activity against it. On the other hand, [18] reported that *Spirulina* extract exhibited stimulation of *Geotrichum* and *Cladosporium* growth. The author suggested that this growth stimulation probably due to *Spirulina* nutritional value properties.

Bioactivity test of alcoholic extract of *Spirulina platensis* against *Candida* spp

Algal extract displayed variable zones of inhibition against tested candida species compared to clotrimazole. In terms to *Candida fameta Spirulina,* extracts exhibited higher inhibition zone (18mm) against it than clotrimazole (15mm). Also, *Spirulina* methanolic extract gave (19 and 20mm) inhibition zones against *Candida lustrans* as compared with (18mm) caused by clotrimazole. No inhibition zone was detected by DMSO-containing well against all tested *Candida* spp. (Figures 4 and 5).

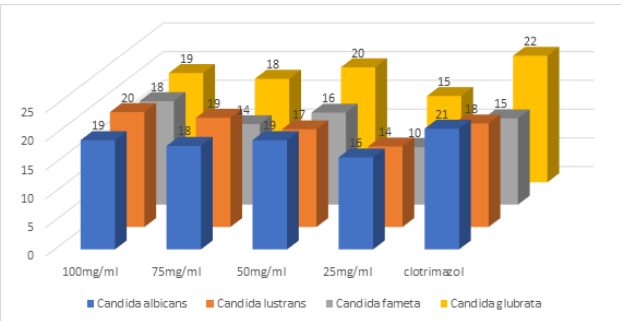

Figure (4) Inhibition zones of different concentrations of *Spirulina platensis* methanolic extracts and clotrimazole against tested *Candida* spp.

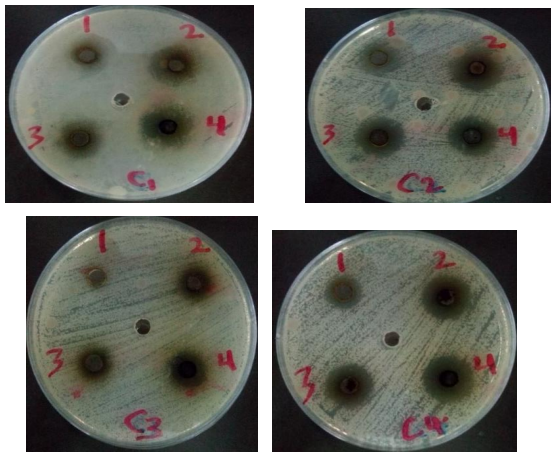

Figure (5) Agar disc-diffusion assay of *Spirulina platensis* methanolic extracts against examined Candida spp. Wells No. C1-4: *Candida albicans,Candida glurbrate,Candida fameta*, *Candida lustrans*, **respectively. Wells No.1-4: algal extracts concentrations (25, 50, 75, 100mg/ml), respectively. Middle well is for solvent (DMSO).**

These tested species of *Candida* may cause serious infections such as progressively common nail infection (Onychomycosis) which is mainly caused by dermatophytes fungi. The disease is extremely difficult to treat. Since that Onychomycosis is not threatening human life, this type of nail infection is an essential public health concern due to its high incidence, low response to therapy as well as suggestive clinical, social and economic impacts [19].

Gas Chromatography-Mass Spectrometry

As a result of GC-Mass analysis, a total of 36 phytochemical constituents were detected for Spirulina alcoholic extract. These compounds possess various biological activities. Eight chemical compounds are authenticated to exhibits antifungal effect. These constituents were listed in Table (1).

Table (1) Antimicrobial chemical constituents identified by GC-Mass analysis

Peak number	Compound name	Retention time	area%	Biological activity	Reference
4	N-Methyl-N-methoxyacetamide	8.722	14.35	Antimicrobial	(20)
5	Octanoic Acid	9.563	9.86	Candidicide, Fungicide, Pesticide	(21)
15	Pentadecanoic acid	19.463	1.14	Antibacterial, anticandidal and antifungal	(22)
17	Eicosanoic acid	21.782	0.83	Antimicrobial	(23)
19	Phytol 2-Hexadecen-1-ol	24.069	0.24	Antimicrobial	(24)
20	cis-10-Nonadecenoic acid	24.523	0.83	Antifungal	(25)
23	Nonanoic acid, 5-methyl-, ethyl ester	25.233	0.37	Stable inhibitor of fungal spore germination	(26)
32	Farnesol	31.737	2.49	Antibacterial, antifungal, antiparasitic	(27)

Several screening studies have been achieved over the past years to record new antibiotic metabolites from microalgae particularly green and blue-green algae)28). *Spirulina platensis* is known to produce a wide range of bioactive molecules, making them a rich natural source of various types of medications (1). This alga showed many therapeutic properties, i.e. the ability to prevent cancer, decreasing blood cholesterol levels, decrease toxicity of kidneys and protection against the harmful effects of radiation (29)..

Conclusions

The data obtained from this study indicated that *Spirulina platensis* alcoholic extracts act as a potential source of antifungal constituents against plant and human diseases caused by fungi. This activity depends on fungal species. GC-Mass data analysis clearly revealed the presence of many documented antifungal chemical constituents. So that, authors suggested that *Spirulina* could be used as an alternative drug to treat *Candida* spp. infections as well as added to food industries to enhance their nutritional value and simultaneously decrease the possibility of food spoilage by molds.

Ethical Clearance: The research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq.

Conflict of interest: The authors declare that they have no conflict of interest.

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