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Biofilm inhibitory potential of *Oscillatoria tenuis* against *Candida albicans*

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Abstract

Prokaryotic autotrophs have a key role in maintaining the sustainability of nature. Their secondary metabolites and stored chemicals have wide utility in human life. Cyanophytes, the primitive producers, can become a necessity of the modern world as they have enormous unexplored features. *Candida albicans*, an opportunistic pathogen having multidrug resistance, fallout health concerns in human and animal hosts. This study focused on the antibiofilm potential of *Oscillatoria tenuis* NTAPD 02, isolated from a hydrocarbon-polluted area against the hyphal switching of *Candida albicans*. Ethanolic extract of the algal sample, OEE, was taken to perform the biofilm quantification test and CLSM studies to determine the antibiofilm potential of *Oscillatoria tenuis* against *Candida albicans*. The MBIC for OEE was found to be 30 µg/mL against *C. albicans* and also shows a 70.8% reduction of fungal biofilm. The GC-MS and FTIR analysis illustrates the presence of potent phenolic hydrocarbons having an anti-proliferative effect. OEE was also found stress generative in *C. elegans* (500 µg/mL). The ROS generation in the worms intensified by increased concentration of OEE. The study proves that *Oscillatoria tenuis,* NTAPD 02, can be considered an anti-proliferative alga against *C. albicans* invasions.

Keywords

Cyanobacteria, BG 11 medium, Confocal microscopy, anti-biofilm, GC-MS, SEM

Introduction

Biofilms are the microbial consortium where the living cells adhere together as colonies by their extracellular polymeric substances on any living or non-living substrate (1). The adhesion of biofilm is a successive course that includes paroxysm of microbes on a surface, adhesion, footing of microbial colonies and biofilm establishment (2). The biofilm helps to overcome intrusive stress and is a pathogenic threat to human beings (3). Biofilms reduce the action of antibiotics and defence mechanisms of hosts and supplement microbial tenacity during prolonged pathogenic infections (4). *Candida albicans* Berkhout is a dimorphic fungus of the family *Saccharomycetaceae* can lead to Candidemia, Candidiasis in mammals, especially in humans and immunity-compromising patients (5). Candidiasis is a critical, superficial infection with an extensive medical spectrum. Oropharyngeal candidiasis occurs in diabetes mellitus, Cancer, HIV 1 or HIV 2 infected patients and organ transplants as they are taking antibacterial antibiotics (6). *C. albicans* usually colonise the epidermis and the mucus layer of the vagina, mouth and intestine and may lead to devious infections (7).

Some of the *Candida* spp. remains unaffected by common antifungal chemicals. Various alga-derived anti-microbial chemicals have been used during the past many years for treating skin diseases of humans (8). Even seaweeds are also identified with many bioactive compounds and possess broad spectrum actions against inflammation (9, 10), anti-fungal (11), antiviral (12, 13), antioxidant (14) etc.

Cyanobacteria are morphologically diverse, photoautotrophic prokaryotes with a wide range of ecological distribution and tolerance (15). They have a prime role in the manufacturing of food, feed, fuel, fertiliser, fine chemicals and pharmaceuticals and their application in pollution control is now being widely studied (16, 17). They perform as ecological indicators and acts as a good bioremediation agent (18). Most of these converters dwell in a nutrient-rich area of their own (19). They can degrade the hydrocarbon and excludes noxious chemicals in soil and the atmosphere near to their habitat (20, 21). Several cyanobacteria, including *Anabaena oryzae* Fritsch*, Anabaena flosaquae* Arm, (22)*, Synechococcus* spp. *(23) Nostoc humifusum* Carmichael ex Bornet and Flahault *(24), Spirulina platensis* Jean Leonard *(25)* etc., produce various intracellular and extracellular active bio compounds against various pathogens. Spirulina, the nutritive alga, can be taken raw and its solvent extracts also act as antibacterial and antioxidant agents. In this study, the ethanolic extracts (EE) of blue-green algae, *Oscillatoria tenuis* Agardh, NTAPD 02, purified from oil spills have been used for the antimicrobial, quorum sensing inhibition and antibiofilm activity against *Candida albicans,* an opportunistic human pathogen.

Materials

All chemicals, utensils and polystyrene plates were sourced from Hi Media, Sigma- Aldrich, Borosil and Greiner Bio-one.

Methodology

2.1 Isolation of the Cyanobacterial strain

Water samples and algal clogs have been collected from the oil spilt place of Tiruchirappalli district of Tamil Nadu, India. These algal clumps were washed three times using deionised water to remove the debris. Different algal groups were analysed using a light microscope and transferred to BG11+ medium (26) and cultured in agar plates by streak and spread method. By repeated streak plating, a pure culture of *Oscillatoria tenuis* was isolated. The BG11+ nutrient medium for cyanobacteria has the composition of: $NaNO_{3-}$ 01.50 g/L, EDTA- 0.001 g/L, $MgSO_4.7H_2O$- 0.075 g/L, $CaCl_2.2H_2O$- 0.036 g/L, Fe $(NH_4)_2(C_6H_5O_7)_2$- 0.006 g/L, Citric acid-0.006 g/L, K_2HPO_4-0.040 g/L, Na_2CO_3- 0.020 g/L, Microelements- 1.000 1 mL/L, pH- 7.8. The algal strain was kept in the white photo-fluorescent lamp, 24 hrs continuously for 25 °C. Identification of the cyanobacteria has been made by using standard manuals and algal flora. The genomic DNA was extracted using Smoker and Barnum method (27).

2.2 Preparation of OEE

The biomass of *Oscillatoria tenuis,* NTAPD 02 was harvested, lyophilised (Penguin Classic plus, Lark Pvt. Ltd, India) and dried. 1 g lyophilised *Oscillatoria tenuis,* NTAPD 02 was powdered using a mortar and pestle. This algal powder has liquefied in 1 mL of ethyl alcohol, filtered in a 0.5 µm diameter filter and allowed to dry in air. 10 mg of air dehydrated sample has been taken and diluted using ethanol: water (Milli Q) at 1:9 proportion (28) and this NTAPD 02 *Oscillatoria tenuis* ethanol extract (OEE) has been taken for further studies.

2.3 Cultivation and Maintenance of *C. albicans*

Clinical isolate of *Candida albicans* obtained from Satyabhama Dental University hospital was cultured in Sebouraud dextrose broth (SDA) agar plates. *C. albicans* was raised for 24 hrs in SDA broth at an rpm of 75 and a temperature of 35 °C before all the experiments. From the broth, 100 µL of the fungal culture, approximately 10^6 cells mL^{-1} had been used for further analyses (29, 30). The average of three independent cultures was used as a single value point.

2.4 Quantitative assays for OEE

2.4.1. *Antimicrobial action of OEE*

Using the disc diffusion susceptibility test and the well diffusion method, the antimicrobial activity of OEE was analysed (31). 24 h old *Candida albicans* raised in SDA agar plates and wells of 5 mm diameter were prepared at the centre of these plates. OEE with different known concentrations was injected in these wells. As a control, 80% ethanol was used and the plates were incubated overnight at 30 °C to observe the zone of clearance (ZoC). Before conducting the crystal violet assay, Spectrophotometric (Hitachi-U 2800) readings at 600 nm were done to observe the effect of OEE on *C. albicans*.

2.4.2 Antifungal study of OEE

This analysis was carried out to check the antifungal effect of OEE against *Candida albicans*. The zone of inhibition is the area where the antimicrobial agent switches off the fungal hyphae in its vicinity. 10 µL of *C. albicans* (10^7 cells mL^{-1}) was poured into microtiter plates (distinct bottom, 24-well polystyrene). With varying 5concentrations of OEE from 10 µg/mL- 50 µg/mL at 10 µg/mL gap addition and fluconazole (10 µg/mL) as a positive control, the minimal biofilm inhibitory concentration (MBIC) of OEE against *C. albicans* was examined for a day at 37 °C. Then the 24-well polystyrene plates were analysed using a spectrophotometer at 570 nm. The concentration on which *Candida* growth was suppressed was taken as the MBIC for the OEE (32). After the biofilm formation process, the *C. albicans* cells and the used media were removed. The cells were thoroughly washed thrice using de-ionized water to remove the unattached cells. The cells were air dried, followed by the staining process for 10 min using a single drop (1 mL) of 0.45% (w/v) of Crystal Violet solution. Afterwards, the dye was discarded. Using non-ionized water, the wells were rinsed twice and de-stained with 1 mL of 100% ethanol. Accordingly, using a

spectrophotometer, the OD value was measured at 570 nm (33).

The formula used for calculating the percentage of inhibition is given below:

% of Inhibition = [Difference between the values of OD-control and OD- test] * 100 / OD- Control at 570 nm

2.4.3 Estimation of Phytochemical composition of OEE

a. Fourier transform infrared spectra

To study the bioactive compound in the OEE, the lyophilised algal powder was taken for FTIR (PerkinElmer Spectrum Version 10.4) analysis, which has a spectrum range between the IR region of 400 cm^{-1} – 4000 cm^{-1}. The spectrum was recorded by coating the OEE in a potassium bromide pellet using the total reflectance technique. The graph was also created using the transmittance mode and the resulting spectra were plotted as intensity against wavenumber.

b. Gas Chromatography- Mass Spectrometry

To analyse the organic active compound in the algal extract, GC-MS was performed. The conditions for the analytical separation of dye-degraded products using the GC-MS (Shimadzu Corporation, Japan 2010) were carried out using HP5MS of 30 m, 0.25 mm i.d., 0.25 µm film thickness capillary column. Then the heat of the GC oven was set to 120 °C for 3 min and gradually raised the temperature to 260 °C at a rate of 6 °C per min. Then, the column heat was raised to 320 ° C at a flow of 8 °C per min for 5 min. 1µL of OEE injected in split-less mode by Helium as the carrier gas at the flow rate of 1.50 mL per min. The temperature of the injector port was set as 280 °C and then the temperature started at 300 °C and 230 °C. The EI mode 70 eV, the emission of 60 mA and 50-500 m/z mass range with a scanning interval of 0.2 seconds were on for the mass spectrometric analysis of OEE.

2.5 Qualitative analysis of OEE

2.5.1. Light microscopy

Qualitative analysis of the anticandidal activity of OEE was performed in static 24-well polystyrene plates (34). Glass slides (1 * 1 cm) swabbed with *C. albicans* were taken in 24-well microliter plates with varying concentrations of OEE (10, 20, 30, 50 and 100 µg/mL). Detachable cells were removed after one day of incubation. Crystal violet dye (0.45% for 5 min) was used for staining the biofilm developed on the glass slide. Subsequently the glass slides were splashed using de-ionized water and dried in air for 5 min. The ability of OEE to inhibit the *Candida albicans* was observed under 400× magnification light microscopy (Leica, DM2000 LED) and the images of the same were captured and developed using LAS 4.9.

2.5.2 Confocal Laser scanning Microscopy (CLSM)

The topographic expressions and the three-dimensional view developed by *C. albicans* biofilm and its response to OEE were analysed using CLSM. The glass slides (1 * 1 cm) with 24 h old fungal cultures developed in 24-well polystyrene plates having OEE added in a gradient manner and raised for a day at room temperature. Plate wells without OEE act as a control. After washing the slides using PBS and staining 0.01% of acridine orange, the same was monitored in Confocal Microscope (710, Carl Zeiss, Germany). For capturing the excited emissions of fluorochrome stained slides, 488 nm laser (Argon) and 480 - 550 nm band pass emission filter used. For studying the biofilm thickness, Z-stacking was done and the 3-D images were captured.

2.5.3 Scanning Electron Microscopy (SEM)

To know the cellular dismantling and morphological disruption of OEE-treated *C. albicans* SEM studies were carried out. A day-old culture of *C. albicans* was cultured and at a rotation of 10,000 rpm centrifuged for 15 min at 4 ° C. The supernatant was collected and washed using one mL saline Phosphate Buffer with pH 7.2. The fungal cells were mounted on 3 mL glutaraldehyde (2.5%) in Phosphate Buffer Saline for 3 hrs and dried out by gradient ethanol of 30%, 50%, 70%, 90% and 100% successively and then air dried. The tests were mounted on aluminium stubs with two-side adhesive carbon tape, then coated with gold-palladium with an ion sputter JFC 1100-coater after drying for 3 hrs. The specimens were inspected using a Scanning Electron Microscope (Carl Zeiss 2011) at 25 kV (35).

2.6. The *in vivo* effect of OEE against wild-type *C. elegans*

Caenorhabditis elegans Maupas, the soil nematode, has been used as a model organism for genetic analysis because of its small size (1.5 mm long adult), molecular switching during its embryological development was much related to many complex animals, including humans, their ease of laboratory cultivation and completes life cycle on 3 -days (36). The wild-type N_2 (Bristol) strains of *C. elegans* and *E. coli* (OP-50) as food supplements were grown in a Nematode Growth medium (NGM). The life span of *C. elegans* was studied by treatment of varying concentrations of OEE against age-synchronized wild-type N2 worm and kept at 20 °C. Following the OEE addition, the worms were observed for inactivity under a light microscope and if they didn't respond to the mild physical touch with the sterile laboratory platinum loop, the worms were measured as dead. Additionally, the effect of OEE on intestinal ROS production was also studied using Confocal Laser Scanning Microscopy.

Results

3.1 Isolation and Identification of NTAPD 02

The cyanobacterial strain *Oscillatoria tenuis* NTAPD 02 isolated from the polluted water has been kept in the National Repository for Microalgae and Cyanobacteria – Freshwater (NRMC-F) India. Its purity was examined on 1.0% Agarose Gel electrophoresis and a single band of high -molecular mass DNA was observed. For amplification, a 16S rDNA fragment was taken for PCR. In a polymerase chain reaction, initial denaturation of template DNA at 94

°C for 2 min, then denaturation was done at 94 °C for 5 seconds, annealing at 47 °C for 10 seconds, elongation at 72 °C for 30 seconds for 40 cycles and the final elongation at 70 °C for 7 min. The amplified product was characterised by gel electrophoresis on 1% agarose gel using 1X TAE buffer at a constant supply of 100V for 45 min and sent for sequencing. The nucleotide sequence was submitted to Gen Bank (NCBI) with accession number MH144154 for *Oscillatoria tenuis* NTAPD02. The bottom-up neighbour-joining method using 16S rRNA gene sequence was taken for Phylogenetic tree construction (Fig. 1).

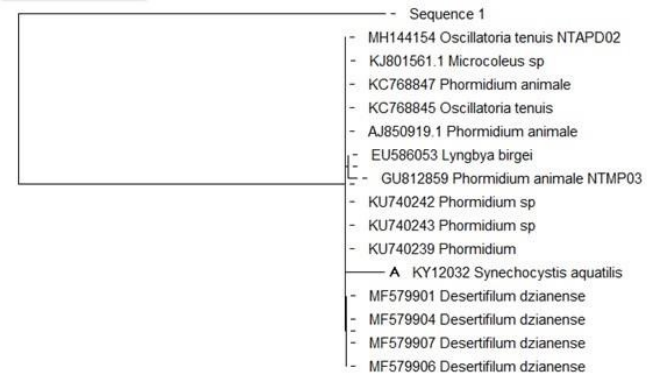

Fig. 1. Phylogenetic tree of *Oscillatoria tenuis* NTAPD 02 using Mega6

3.2 Determination of the antibiofilm activity of OEE

The antimicrobial activity of the OEE was minimum as it forms a negligible zone of inhibition only when compared with fluconazole. But the algal extract has an inhibition effect on *C. albicans* during biofilm formation. The biofilm thickness of *C. albicans* was reduced by the increasing concentration of algal extract. The maximum decrease in cell sustainability has been viewed at 30 µg/mL of algal extract. The ring test was initially performed to check the anti-biofilm activity of the algal extract. 1 mL of SDA broth (Hi media) was suspended with 20 µL of the fungal cells and the OEE with varying concentrations of 10, 20 and 30 µg/mL was added and incubated at 37 °C for a day. To find out the minimum biofilm interruption concentration (MBIC) of OEE, the growth absorbance of *Candida albicans* was observed at 570 nm for 2 days and then the biofilm inhibition percentage was determined with the help of the formula. From the quantitative value, it has been evaluated that the BIC for OEE against *C. albicans* was found to be 30 µg/mL, where it shows maximum inhibition of 70.8% of fungal biofilm (Fig. 2).

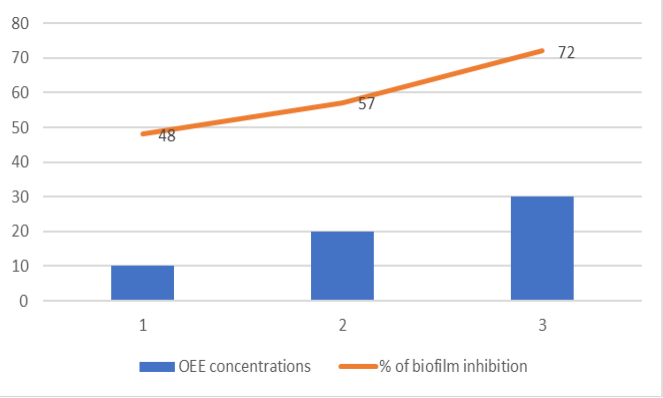

Fig. 2. Biofilm inhibition assay for OEE, against *C. albicans.*

3.3 Microscopic studies

3.3.1. Light microscopic assay

The control glass slide containing pure *C. albicans* biofilm (shown as A in Fig. 3) and OEE-treated slides with 10 µg/mL, 20 µg/mL and 30 g/mL showing a reduction of biofilm as 48%, 57% and 72% respectively in B, C and D of Fig. 3. The antibiofilm activity of OEE was further confirmed by confocal laser scanning microscopy analysis.

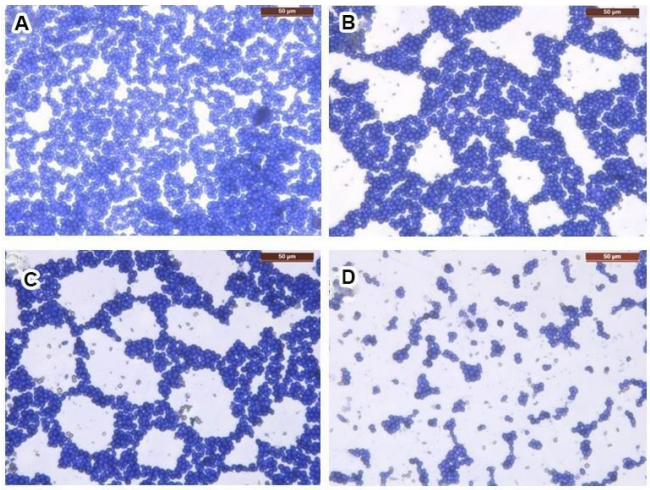

Fig. 3. Light microscopic images showing the antibiofilm activity of OEE against *C. albicans*
A- Control, B- 10 µg/ml C- 20µg/ml D- 30 µg/ml OEE treated and stained by crystal violet.

3.3.2 Scanning electron Microscopy

The SEM images show the external interaction of OEE on the fungal cells (Fig. 4). The control cells had an uninterrupted biofilm of *C. albicans* and the OEE-treated slide shows *C. albicans* biofilm disruption. From the images, it was clear that the treated cells have lesions in their cell wall and connections with neighbouring cells are vanishing.

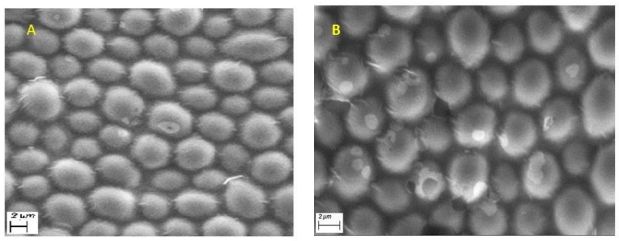

Fig. 4. SEM images showing the effect of OEE with *C. albicans*
(A)- Control with C. albicans and (B) with OEE

3.3.3 Confocal laser Scanning Microscopic images (CLSM)

CLSM was used to study the Z- stacking and 3-D images and the mean intensity of laser emission as the power gained is equivalent to the thickness of biofilm present in the slide. The confocal laser emission-excitation studies on *C. elegans* mounted in slides and OEE-treated glass slides stained with acridine orange (0.1%) in the wavelength of 480 nm show a considerable decrease in the biofilm concentration of fungal cells with an increasing amount of OEE (Fig. 5). The control slides, where the fungus alone have an intensity emission of 82.744 while the OEE treated slides decrease its light emission by hiking OEE concentrations. At 30 µg/mL of OEE extract, the intensity is

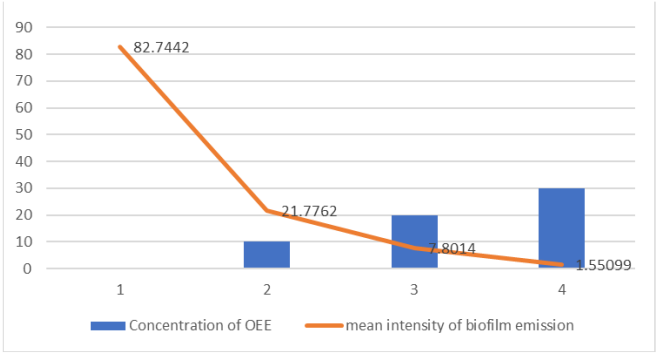

Fig. 5. CLSM - Excitation intensity graph of *C. albicans* at various OEE concentration

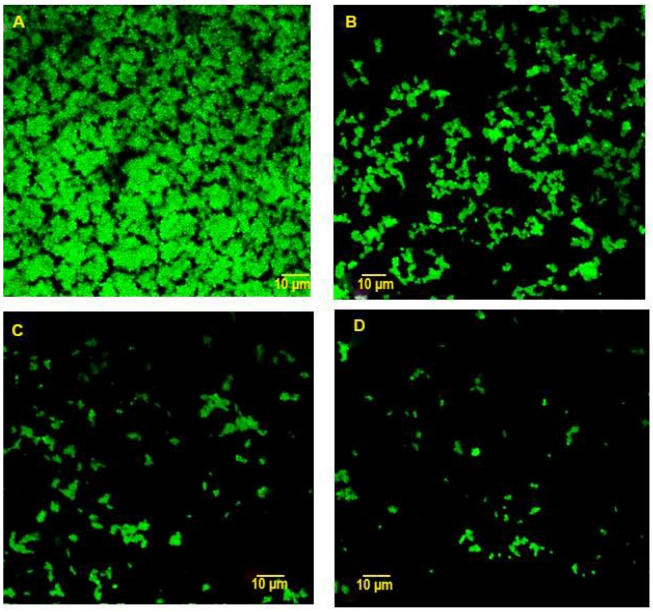

Fig. 6. CLSM-2-D images of the biofilm interruption of OEE against *C. albicans*

A- the control, *C. albicans* in PBS, B, C, D - *C. albicans* treated with 10,20, 30 µg/ml of OEE showing consecutive decrease in biofilm of fungus.

coming near zero. As light intensity is directly proportional to biofilm thickness, it is clear that the biofilm was vanishing when the OEE extracts were increased.

Further, the CLSM images also emphasise the biofilm interruption by OEE addition. The decrease in biofilm by the increase of OEE shows the antibiofilm effect of NTAPD 02 (Fig. 6). The 3-D images also show the interruption in biofilm thickness (Fig. 7).

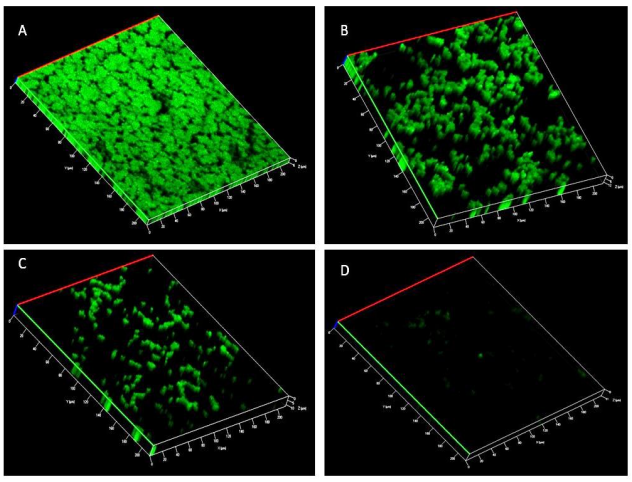

Fig. 7. 3-D CLSM images of *C. albicans* against various concentration of OEE A-control and B, C, D- algal treated fungal slide with 10,20,30 µg/ml of algal extracts.

3.4 NTAPD 02 antibiofilm chemical characterisation studies

3.4.1 FT-IR

To evaluate the biofilm interruption and changes in chemical constituents that occurred in the aggregation of fungal cells, FTIR analysis has been done. From the graphical peaks, it can be noticed that the control having C=O stretches in the 1866 cm^{-1} has been changed to aliphatic C-H stretches in the 1022 cm^{-1} range (Fig. 8).

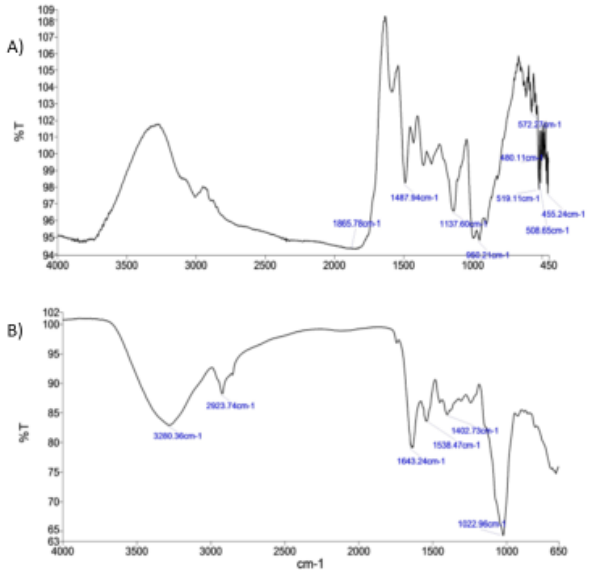

Fig. 8. FT-IR showing the (A)- *Candida albicans* (B)- OEE treated *Candida albicans*

3.4.2. GC-MS analysis

The gas chromatographic analysis of the OEE (Table 1), shows the presence of the anti-proliferative compound Naphthalene at an area percentage of 12% and the other compounds having maximum area were Tetra decanoic acid, Neophytadiene, Hexadecenoic acid, 2-Hexadecanoic acid, Linoleic acid ethyl ester, Ethyl oleate, 9-Octadecanamide, Octadecanoic acid, 1,2-Benzene dicarboxylic acid and Stigmast-5-en-3-ol respectively. Of these volatile oils, the decanoic, linoleic compound shows the presence of algal or essential oil and most of the other compound has some antibiotic properties (Fig. 9).

3.5 Effect of OEE on the *C. elegans*

OEE with different concentrations of 10, 50, 100, 250 and 500 µ g/mL was added to the NGM media where the nematodes were raised. The OEE with minute concentration doesn't affect on worms, so with higher concentrations, 250 and 500 µ g/mL proceeded for further studies. The number of worms that survived in each plate was counted and the survival graph (Fig. 10) was constructed to study the effect of OEE on the survival rate of *C. elegans.* The total life span of the worms was about 25 days and the OEE has a little anti-proliferative effect on the growth of worms. The observation showed that increasing concentration of OEE induces intestinal ROS production (Fig. 11). This may be the result of physiological stress exerted by phytochemicals in OEE to the growth of worms.

Table 1. Phytochemicals present in OEE after GC-MS analysis.

Peak	Retention time	Area %	Height %	A/H	Compound
1.	4.359	1.19	1.65	1.37	Oxime-methoxy-phenyl
2.	8.314	12.91	8.65	2.84	Naphthalene
3.	12.344	1.07	1.11	1.84	Decahydro-4-Quinolinol
4.	13.561	2.31	2.54	1.73	Ethyl pentadecanoate
5.	15.493	5.14	3.8	2.58	Tetra decanoic acid
6	16.293	1.75	1.8	1.85	Neophytadiene
7.	17.861	7.63	8.96	1.62	Hexadecenoic acid
8.	18.991	4.71	5.47	1.64	2-Hexadecanoic acid
9.	19.442	6.19	7.4	1.59	Linoleic acid ethyl ester
10.	19.5	8.57	10.09	1.62	Ethyl oleate
11.	19.72	1.84	2.15	1.62	Octadecanoic acid
12.	21.204	4.24	4.21	1.92	9-Octadecanamide
13.	22.58	6.16	7.07	1.66	1,2-Benzene dicarboxylic acid
14.	24.321	4.74	2.03	4.45	Stigmast-5-en-3-ol

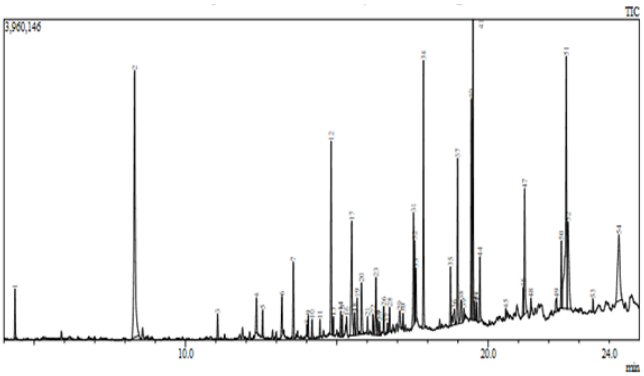

Fig. 9. GC-MS peaks showing the organic compounds in OEE

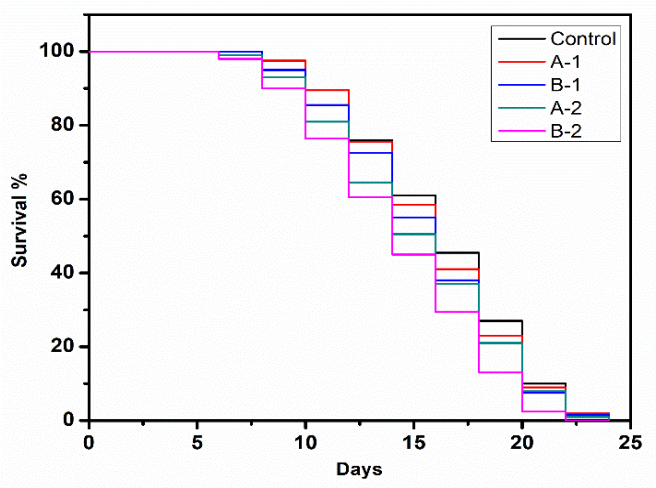

Fig. 10. The effect of OEE on Survival of *C. elegans* N2 worms

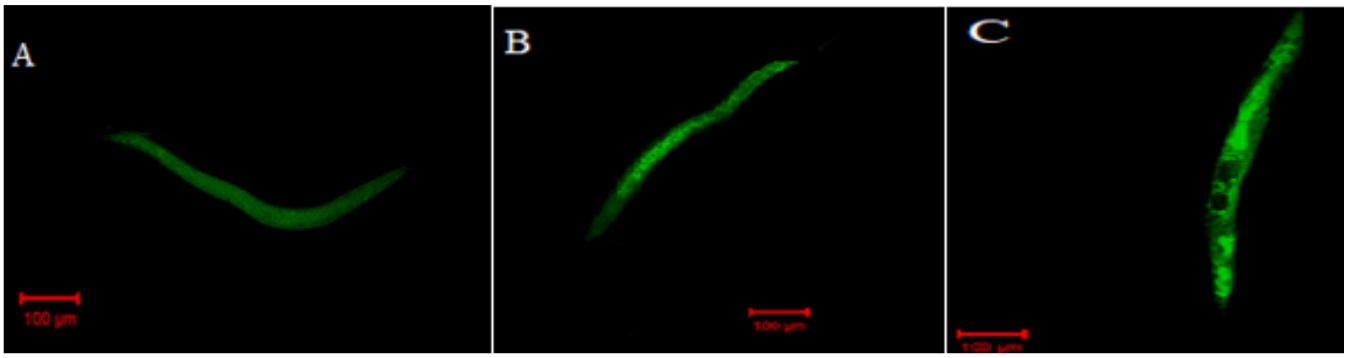

Fig. 11 A,B,C. Showed the induction of intestinal ROS production in wild-type nematodes

Discussion

Epidermal infections, especially Candidiasis caused by *Candida albicans*, is a common fungal infection. *The cli*nical antibiotic therapy used now for fungal diseases was facing the challenge of new antifungal drug resistance strains. In this context, nutraceutical algae being renewable resources have a great promotional value. The fungal establishment, especially as biofilm, can be seen in the early stage of infections. The biofilm mode of development manures the growth of the pathogen from antimicrobial compounds (37). This study proved cyanobacteria, *Oscillatoria tenuis* acts efficiently on *C. albicans* proliferation. Even in a small concentration of the algal extract (30 µg/mL), a remarkable inhibition over the *Candida* growth was observed. In all the microscopic studies, it was evident that the treatment of algal extract

reduced the fungal biofilm. The OEE concentration is affecting the growth of *C. elegans* and the longevity of the worm is reduced to 23 days than the normal 25 days. OEE also induces the stress factor in the worm and leads to ROS production in the intestinal part of the worm.

In recent years, using marine algae to cure fungal diseases has been getting noticed. Reduced side-effects and cheap production costs make it eco-friendly and easy to rear. Further, this algal study has been carried out in animal experiments to study the possibility of using this alga against the human epidermal pathogen-*Candida* (38). Certain animal studies on oral cancer have shown positive resistance to algae. The potential of the algae *Spirulina fusiformis* Gomont, to prevent precancerous mouth sores has also been studied in humans (39). Also, many compounds extracted from seaweeds possess anti-microbial and anti-inflammatory activities (40, 41). The ethanolic extract of *Oscillatoria tenuis*, NTAPD 02, possess anti-proliferative property and the potential to inhibit candida biofilm growth through its broad-spectrum phytocompounds. Based on these preliminary findings, large scale-controlled studies on the extract of *Oscillatoria tenuis*, NTAPD 02 active principles may be carried out.

Conclusion

Blue-green algae are prokaryotic organisms and play an important role in converting nutrients in nature. Besides, they have a lot of clinical and nutraceutical importance and they can be utilised properly. As they are fast growing and the mass multiplication is higher, they can be used commercially. The study showed an anticandidal effect of *O. tenuis* and its toxicity against *C. elegans*. The ethanolic extract of algae in minute quantity itself can switch off the fungal biofilm. The study proves the anticandidal effect of *O. tenuis* even at a minute level.

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Authors contributions

DP collected the sample, isolated and made a pure culture of it. Quantitative and qualitative studies, genetic studies of the specimen were also carried out and drafted the manuscript. NT did the identification, genomic submission in NCBI, give overall guidance and reviewed the manuscript. Both the authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

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