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Molecular identification and Anticandidal Activity Assessment of *Desmodesmus subspicatus* Against Multidrug-Resistant Candida Isolates from Intensive Care Units

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ABSTRACT

Desmodesmus exhibits significant morphological diversity, leading to multiple synonyms being assigned to this species based solely on optical microscopic morphology. Prior studies have demonstrated that *Candida* spp. displays increased resistance in critical care units relative to non-ICU isolates. In the current study, the green alga *Desmodesmus subspicatus* (Scenedesmaceae) from AL-Hilla River in Iraq was isolated, purified, and identified morphologically via optical microscope and at the level of molecular using 5.8S ribosomal RNA genes, as well as the isolate species recorded in NCBI gene bank with PQ192620.1. Using the MEGA and BLAST programs, we constructed a phylogenetic tree and analyzed the similarities and differences with international isolates. The results showed that the antifungal activity of the alcoholic extract of the algae *Desmodesmus subspicatus* against *C. dubliniensis* had a standard deviation of 4.66 and a mean inhibition zone of 23.90. The average inhibition zone against *Candida albicans* was 25.23, with a standard deviation 5.54. *C. glabrata* had an inhibition zone with an average of 24.20 and a standard deviation of 4.04.

1. Introduction

Desmodesmus was recognized as a distinct genus apart from *Scenedesmus* by An *et al.* (1999). The genus *Scenedesmus* was first described around two centuries ago (Meyen,1829), and a significant number of species have been identified solely based on morphological features. (An *et al.* ,1999) conducted a comprehensive examination utilizing molecular phylogenetic and structural examination of internal transcribed spacer 2 (ITS2) sequences. Their findings demonstrated that *Desmodesmus* is a distinct genus apart from *Scenedesmus*. *Desmodesmus* displays significant morphological diversity in response to environmental factors. Consequently, relying purely on optical microscopic morphology for descriptions has resulted in the assignment of multiple synonyms to this species (Demura,2024). *Desmodesmus* exhibits a mostly colonial (coenobial) shape consisting of four linear cells. Notably, the outer cells possess a distinct structure called a "spine" at their periphery. Nevertheless, the spine may be lacking in some species or present in every colony cell (Ye, *et al.,*2020; Premaratne *et al.*,2021). Microalgae are highly regarded as a prime source of valuable materials for producing pharmaceuticals, nutraceuticals, and cosmetic items(de Oliveira *etal.,*2022). Algae also have the potential to serve as a rich supply of vitamins, minerals, and protein for human consumption (Dantas and Oliveira, 2019). Numerous bioactive substances, including amino acids, alkenes, steroids, phenolic compounds, carotenoids, terpenoids, and cyclic polysulfides, are found in microalgae (Eze *etal.,*2023). These chemicals exhibit several physiological activities, for example, Antibacterial, antiviral, antioxidant as well as antifungal activities (Rashed *et al.,*2023). With the increase in the recorded cases of antibiotic resistance the issue of concern becomes global. To address the problem, it is crucial to make an emergent search for the new antibiotics and other chemicals with antimicrobial activity (Yap *et al.*, 2019).

Candida species is an opportunistic fungus that causes skin, mucosal surface and systemic tissue infection. Some attributes of virulence of *Candida* spp include; adhesion, ability to form a biofilm, the production of hydrolytic enzymes, and antifungal drug resistances; Mba and Nweze, 2020. The infections by Candida have shifted from being relatively inconsequential to becoming a significant source of morbidity and mortality, El-Ganiny and his team conclude (El-Ganiny *et al.,* 2021). The four classes of antifungals for *Candida* infection include polyenes, azoles and the latest, echinocandin (Wall and Lopez-Ribot, 2020). Multidrug-resistant Candida which is resistant to multiple antifungal drugs is a global concern (El-Ganiny *et al.,* 2021). A major concern in human health is antimicrobial resistance or AMR which occurs when bacteria, virus, fungi and parasite develop and cease to respond to chemotherapeutic agents. It also becomes difficult to address sickness and even to spread out communicable diseases, among them. As such, these agents resulted in higher hospital patient mortality rates to increase the overall costs of human health due to expensive medication and longer hospital stays. In the course of World Antimicrobial Awareness Week 2020 WHO noted that increased use of antimicrobials in the COVID-19 epidemic caused by SARS-CoV-2 could contribute to faster development and spread of microbial resistance. Thus, timely emergence of enhanced and effective solutions is of enormous importance in relation to the question of combating resistance (Chuy *et al.,* 2022). In Iraq, some studies have investigated the effectiveness of algal extracts against *Candida* , dermatophytes, and phytopathogenic fungi. Dwaish (2019) studied the inhibitory effectiveness of the alcoholic extract of the algae *Cladophora glomerata* and *Oedogonium* sp against some dermatophytes. Khaleel & Dwaish, (2023) studied the efficacy of the hexanoic extract of *Spirogyra* against the fungus *C. albicans*. The efficacy of the ethanolic extract of the algae *Chlorella vulgaris* and *Oscillatoria limnetica* against the fungi *Candida albicans and Candida glabrata* was tested by Nesrullah *et al*., (2023). The efficacy of the alcoholic extract of the algae *Spirulina platensis* against the fungus *Aspergillus niger* was tested by Farkha (2023).

The present study was aimed to isolate, purify,

as well as characterizing the algae *Desmodesmus subspicatus* at the level of phenotypically and genotypically, finally studying the microalgal extract activity “for the first time in Iraq” against some resistant *Candida* strains isolated from the intensive care unit.

2. MATERIALS AND METHODS

2.1. Algae sampling and cultivation

Several liquid algal samples were obtained from AL-Hilla River in Iraq (GPS: 32°33'51.5"N 44°23'50.8" E) from October to November 2023. We promptly sent the specimens to the biology department laboratory at the College of Education, Karbala University, to isolate and cultivate the green algae species using the dilution technique first described by Stein, (1997). Combined 1 mL of water sample with 9 mL of distilled water and centrifuged at 4000 rpm for 10 minutes. Diluted the sediment with 10 ml of distilled water to identify the green algae species using a novel optical microscope, and produced slides from this prepared mixture. Monoalgal cultures of green algae species were obtained by using 5ml of the algal mixture and pelleting the algal cultures several times with distilled water in a CNWTC, 4000 rpm, 10 min. Subsequently, the washed samples of 1 ml from each sample were transfer into sterile test tube and the volume brought to 10 ml using sterile Chu-10 liquid medium. The cultures were placed in a growth chamber at a light-and-dark cycle ratio 14:10 (Stein, 1997).

2.2. Microalgae cultures purification

According to Wiedeman *et al* (1964) who provided the method of getting axenic cultures of microgreen algae. First, the different cultures of algae were washed independently with sterile distilled water in order to isolate them by applying centrifugation at 4000 rpm for 5 minutes. After that, to obtain completely an axenic algal cultures, the last sediment was washed with sterilized distilled water 12 times. For a duration of 5 min. Axenic algal cultures were obtained by washing the resultant sediment with sterilized distilled water and repeating the process 12 times. These cultures were subjected to tests which would rule out bacterial and fungic contents and contamination by other microbes as noted by Stein, (1997).

2.3. Morphological identification

According to John *et al*. (2002), (Soylu and Gönülol,2012), (Lortou and Gkelis,2019), (Lortou *et al.,*2022), and (Demura,2024) The cell count per coenobium, the cell shape, and the cell structure was described, as well as the taxonomic identifications were performed finally.

2.4. Molecular analysis employing polymerase chain reaction

The DNA was extracted from the liquid culture of the isolated and purified algae using the ZR Fungal/Bacterial/Yeast/Algae DNA MiniPrep kit. Electrophoresis and nanodrop spectrophotometer are used to check the concentration and purity of DNA (Sambrook *et al*., 1998). The NanoDrop spectrophotometer was used to determine and assess the purity of the extracted DNA sample at a wavelength of 260/280 (García-Alegría *et al*., 2020).

Investigation primers are supplied by Integrated DNA Technologies company, based in *Canada*, (Forward: 5' (TCC GTA GGT GAA CCT GCG G) 3 , Reverse: '٥ (TCC TCC GCT TAT TGA TAT GC) 3 ').

Several experiments were conducted by performing the Gradient PCR test to determine the optimal reaction conditions for the initial denaturation and primer annealing for DNA amplification. The concentration of the DNA template (1.5-2µl) was also changed. The reaction conditions for the initial denaturation were represented by a temperature of 94°C for one cycle and for 5 minutes. The same temperature was used for the final denaturation for 40 seconds during 35 cycles. The primers were annealed to the template at a temperature of 52°C for 35 cycles and for 40 seconds. The extension was performed at a temperature of 72°C, with the initial extension occurring over 35 cycles for 40 seconds, while the final extension took place over one cycle for 7 minutes.

Conducted conventional PCR testing using the Maxime PCR PreMix Kit (i-Taq) (Intron, Korea). To obtain a final volume of 25 µL, 1.5 µl of DNA was transferred into two tubes, and then the rest of the volume was filled up to 25 µL by adding deionized water.

Sequences of the molecular products of *D. subspicatus* DNA were resolved by The

Macrogen Company. conducted a detailed investigation and analysis of the isolated sequences to search the National Center for Biotechnology Information (NCBI) database. In this work, adopted the comparison analysis that enabled determining the similarities and differences between the algae in our study and those throughout the world by using NCBI. By means of MEGA we were able to design an evolutionary tree for the organism *D. subspicatus.*

2.5. *D. subspicatus* Methanolic Extract

The biomass of *Suspicious* was collected through centrifugation at 4000 rpm for 10 minutes. The biomass was washed at least four times with deionized water. After the biomass was subjected to maceration with 250 ml of methanol for 7 days with 10 grams of dried biomass. Biomass was mixed with 250 ml of methanol and subjected to maceration for a duration of 7 days. The mixture was then made to stand in complete darkness and at room temperature as well. The extract was filtrated with Whatman paper and after that the mixture underwent 18 min of centrifugation with the speed 4000 r.p.m. The clear extract was evaporated by air and desiccated by means of air subsequently, finally 500 mg was collected and dissolved in 5 µl of Di-methyl sulphur dioxide (DMSO) (Makhlof *et al.,* 2023).

2.6. Anticandidal activity test

the obtained extract of *D. subspicatus* were used for evaluating its antifungal activities against three species collected from male patients in the Intensive Care Unit (ICU) at Hilla Educational Hospital. Samples were collected from patients displaying clinical symptoms of oral candidiasis, including white spots on the surfaces of the oral cavity and red patches on the palate (Talapko *et al.,*2021). In order to prepare inoculums for testing, *Candida glabrata, Candida dubliniensis*, and *Candida albicans* were subcultured on Sabouraud's dextrose agar (SDA) medium (Accuamix, India) and incubated at 37°C for 24 hours (Khalaf *et al.,*2021). The agar well diffusion technique was used to evaluate algal extracts antifungal activity to inhibit fungi growth. An inoculum was prepared by suspending two to three colonies from a 24-hour-old culture in 10 ml of a 0.85% sodium chloride solution. Turbidity was calibrated to 0.5 McFarland standard units. Prepare a set of methanolic alga extracts in DMSO at 10, 20, 30, 40, and 50 mg/ml concentrations after sterilizing the solution with a 0.22 µm syringe filter. Each well contained 100 µl of the methanolic extracts, while the negative control was 100 µl of DMSO, and fluconazole was utilized as a positive control of 10 mg/ml (El-Saadony *et al.,*2021).

Utilizing the microdilution technique to determine the minimum inhibitory concentration (MIC) by specifying the lowest dose of antimicrobial agents that successfully prevented the visible growth of microorganisms, together with the minimum fungicidal concentration (MFC), which is the lowest concentration of antimicrobial agents that resulted in the death of 99.9% of the inoculated organisms (El-Saadony*etal.,*2021). The MIC and MFC values of Fluconazole and algal extract were specified following Bhargava's (2019) method at concentration ١٦٠٠µg/ml.

2.7.Statistical analyses

The averages of the inhibition zone and standard deviation were calculated using the SPSS program.

3. RESULTS

By means of light microscope regarding morphological feature, our obtained isolate from Al-Hilla River in Iraq described completely as below :

Phylum: Chlorophyta
Subphylum: Chlorophytina
Class: Chlorophyceae
Order: Sphaeropleales
Family: Scenedesmaceae
Subfamily: Desmodesmoideae
Genus: *Desmodesmus*
Species: *Desmodesmus subspicatus*

Their morphology was oval and elongated, with a solitary, conspicuous pyrenoid and cup-shaped Chloroplast. Despite the prevalence of two-celled coenobia, the majority consisted of four-celled coenobia, which consistently exhibited lateral spines in both the outer and inner cells. The cell had dimensions of 5–11 micrometers in length and 3–6 micrometers in breadth, as shown in Figure 1. The DNA was obtained from the algal biomass with a concentration of 25.3 ng/ml and a

purity of 1.824. We employed ITS replication sequences to distinguish between the genera *Desmodesmus* and *Scenedesmus*. A 275 base pair linear DNA was generated by isolating and replicating 5.8S ribosomal RNA genes from *D.subspicatus*. Identification of sequences of *D.subspicatus* indicating genetic connection with isolates from Spain, China, Jordan, Taiwan, India, and Germany has been achieved. Table 1.

Table 1: Comparison of the 5.8S ribosomal RNA gene sequences of the *Desmodesmus subspicatus* strain with those of other strains documented globally at NCBI.

ID	Origin	Isolate	Identities	Query Length	Query Cover
PQ192620.1*	Iraq	*D.subspicatus*	100%	275 bp	-
MT462646.2	Spain	*Desmodesmus* sp	97.46%	661 bp	100%
MT462647.1	Spain	*Desmodesmus* sp	97.46%	638 bp	100%
OL409186.1	China	*D.subspicatus*	97.10%	694 bp	100%
MW130883.1	Jordan	*D.subspicatus*	97.10%	597 bp	100%
JF835989.1	Taiwan	*Desmodesmus* sp	97.10%	619 bp	100%
MN864182.1	India	*Desmodesmus sp*	97.10%	586 bp	100%
MK975481.1	Germany	*D.subspicatus*	97.10%	1472 bp	100%
JF835990.1	Taiwan	*Desmodesmus sp.*	92.83%	648 bp	100%

We documented *D. subspicatus* (PQ192620.1) in the GenBank database, evaluated the nitrogenous bases, and compared them with some closely related species worldwide using the BLAST tool. The linear DNA of *D. subspicatus* shows a high degree of similarity, 97.46%, to the species from Spain, with Query Cover 100%. Therefore, the dissimilarity can be attributed to 7 different nucleotides. With a Query Cover of 100%, the similarity to the species from Taiwan is 92.83%, indicating that the dissimilarity may be attributed to 20 different nucleotides. Table 2

Table 2: Differentiations of species closely associated with *D.subspicatus*

Query	Subject	Type of variation	Location	Nucleotide
D.subspicatus PQ192620.1	*Desmodesmus* sp MT462646.2	Deletion	69	A
		Substitution*Transversion*	277,292,328	T\A, T\G
		Substitution\ Transition	242, 309,320	A\G
	Desmodesmus sp. JF835990.1	Deletion	77,104,11,112	A, A, C, T
		Substitution*Transversion*	107,118,126,152,153, 172,286,301,337	A/C, G/T, T/A, A/C T/A, G/T,A/T,A/T,G/T
		Substitution\ Transition	79,154,159,180,251,318, 329	T/C, T/C,T/C,A/G,G/A, A/G,G/A

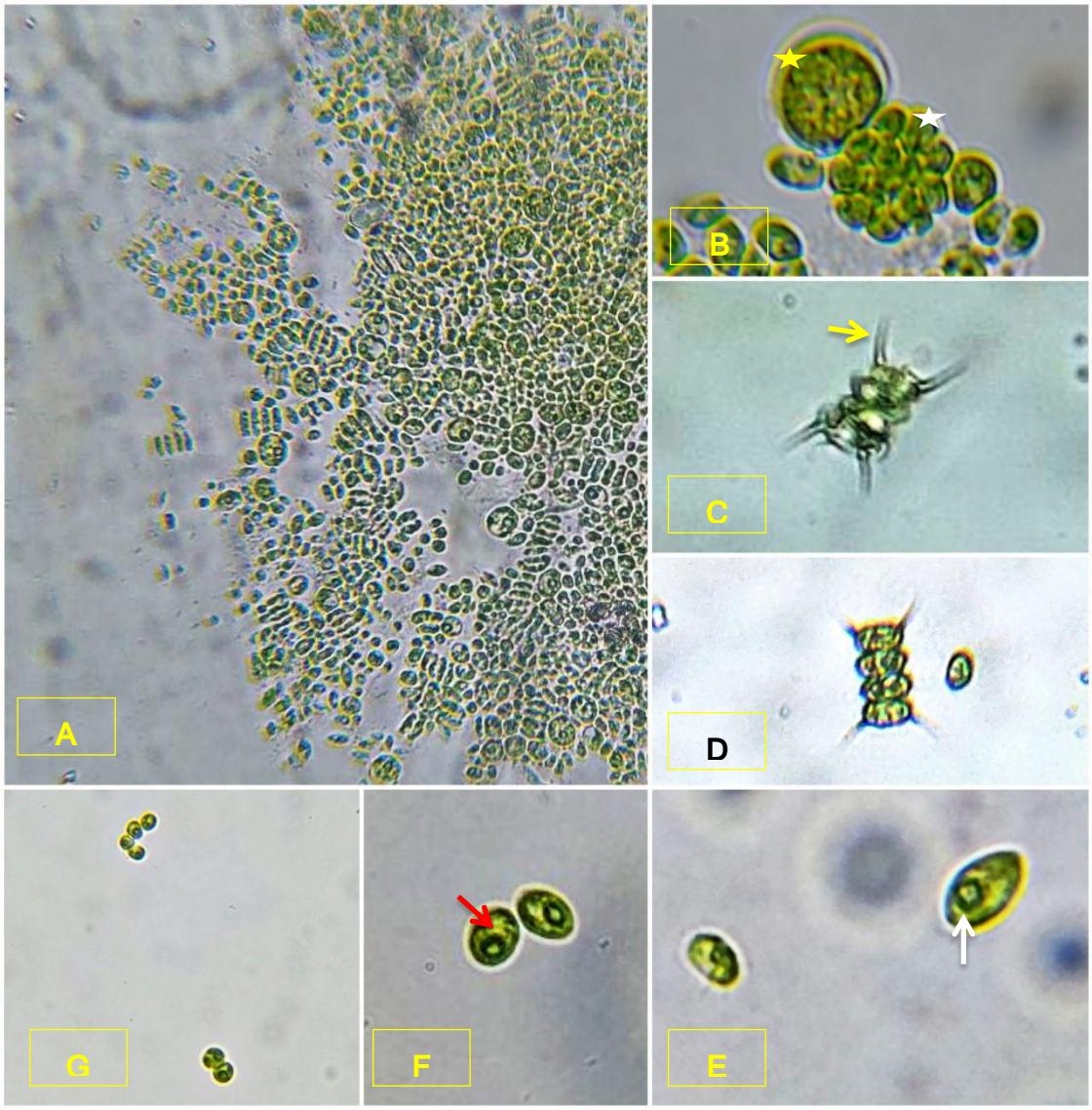

Figure 1: Phenotypic identification by light micrographs of Desmodesmus *subspicatus* . A: 40X of *D.subspicatus* Cells and colonies. B:100X, Asexual reproduction: asexual reproduction: Autosporangium (Yellow star) and releasing autospores (White star). C, D : 100X, Four-celled coenobia with lateral spines (Yellow arrowhead). E: An oval elongated cell with a solitary big pyrenoid (White arrowhead). F: 100X, A cup-shaped chloroplast (Red arrow). G: 100X, Four and two-celled coenobia.

By comparing the nitrogen base sequences of identical genes using molecular evolutionary genetics analysis (MEGA), we can identify evolutionary relationships among the same strains and calculate the genetic distance by neighbor-joining. The phylogenetic relationships were created based on the ITS-rDNA nitrogen bases and global ones of the same strains Figure 2.Table 3 shows that the methanolic extract of *D. subspicatus* showed very promising inhibitory zones against all tested pathogens, with activity levels ranging from moderate to high. We found substantial antifungal activity levels against *C. dubliniensis*, with a mean inhibition zone of 23.90 and a standard deviation of 4.66. Against *C. albicans*, the mean inhibition zone was 25.23 with a standard deviation of 5.54. The mean inhibition zone of *C. glabrata* was 24.20, with a standard deviation of 4.04. In comparison to multi-drug resistant species, the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of algae extract and fluconazole were found to be 0.3905, 0.781 µg/ml, and 0.003, 0.006 µg/ml, respectively, in *C. albicans*.Table 4 . At a fluconazole concentration of 10mg/ml, *C. dubliniensis* developed colonies in the inhibitory zone and displayed heteroresistance.

Table 3: Assessment of the antifungal properties against *Candida*

Candida spp	10 mg/ml Fluconazloe	Extract	Mean zon 20 mg/ml
C. glabrata	29.00	20.00	20.5
C. dubliniensis	33.00	18.5	20.00
C. albicans	35.00	20.00	25.00

Candida spp	Mean zones of inhibition					
	10 mg/ml		20 mg/ml	30 mg/ml	40 mg/ml	50 mg/ml
	Fluconazloe	Extract				
C. glabrata,	29.00	20.00	20.5	24.00	27.5	29.00
C. dubliniensis	33.00	18.5	20.00	24.00	28.00	29.00
C. albicans	35.00	20.00	25.00	29.00	29.00	35.00

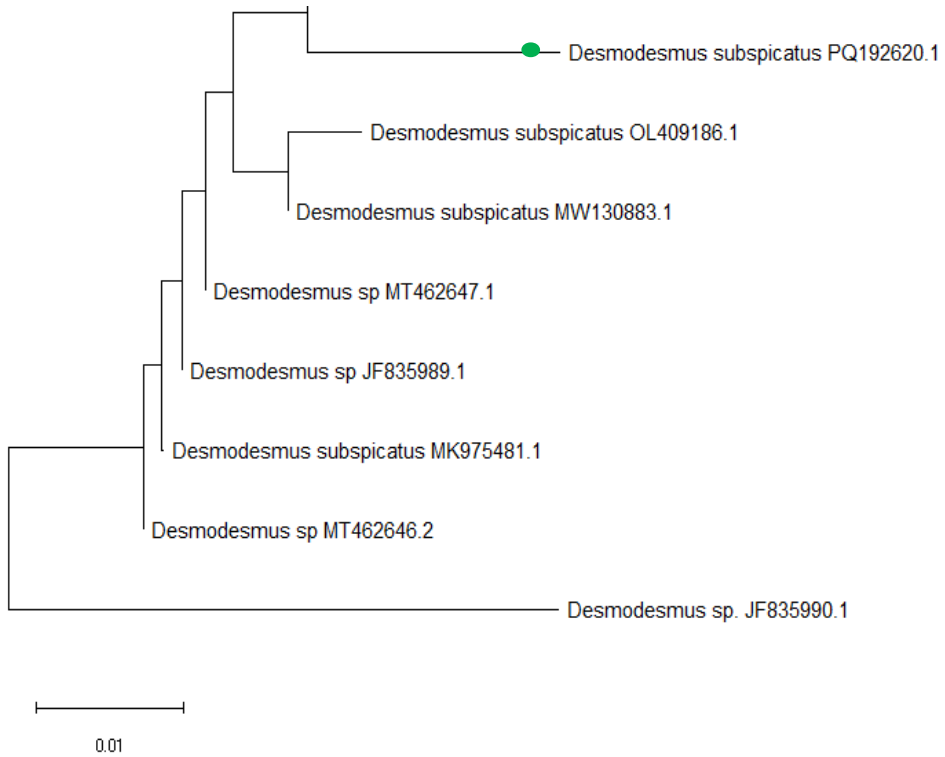

Figure 2: Phylogenetic tree of *D. subspicatus* (Distinct green point)

4. DISSCUSION

The genetic diagnosis of the alga *D. subspicatus* was conducted, in addition to the microscopic diagnosis. The taxonomic definition of *Desmodesmus* species is challenging due to their phenotypic flexibility. Numerous morphological features, which could serve as taxonomic markers, need to be discernible with the light microscope or exhibit instability. The variability of spine number and spine length within a clone renders them unsuitable as fixed characteristics for taxonomic comparison. Hundreds of clones have demonstrated this phenomenon (Demura,2024). Hence, the molecular features are valuable as they exhibit a more consistent pace of evolution compared to morphological features. Thus, culture and sequencing analysis is the most straightforward approach for species identification within this genus (Fawley and Fawley,2020). The nitrogen bases of the Internal Transcribed Spacer (ITS) act as a universally unique barcode that exhibits significant diversity and preserved positions. Consequently, they are very suitable for designing universal primers. An extensive collection of Internal Transcribed Spacer (ITS) copies from various strains is available in the GenBank database, which greatly simplifies the comparison process (Tamura *et al.,*2021).

Recent decades have seen a growing focus on novel natural antimicrobial chemicals driven by shifts in consumer tastes and the proliferation of resistant microorganisms. The administration of commercial antibiotics to treat human diseases

results in unfavourable adverse effects. Potential bioactive chemicals of interest in the pharmaceutical business may include cell extracts and active components derived from different types of algae (Afzal *et al.,*2023). The present study results indicate that the methanolic extract of *D. subspicatus* exhibited highly effective inhibitory zones against all examined pathogens . The results of the methanolic extract activity of the green algae *D. subspicatus* did not agree with the results of the methanolic extract of the green algae *Micractinium reisseri*, which was studied but at lower concentrations (50 µg/ml) against the fungus *C. albicans* by Makhlof *et al*. (2023). However, the study results agreed with Musbah H *et al*., (2012), who studied the efficacy of the methanolic extract of the green algae *Ulva lactuca* against the fungi *C. albicans* and *C. glabrata* using higher concentrations (250 mg/ml). However, Saidani K. *et al*. (2019) found that the same algae, *U. lactuca*, were positive with lower concentrations against the fungus *C. albicans*. The study results also aligned with the findings of Ahmed *et al*. (2020) regarding the methanolic extract of the marine green algae *Halimeda opuntia*, which was effective against the fungus *C. tropicalis* at a concentration of (3 mg/ml)

In the current study, the fungus C. glabrata showed high resistance to the algal extract and fluconazole, with minimum inhibitory concentrations of 200 and 100 micrograms/ml, respectively. There are evidences suggesting that non-sensitivity and mortality rates detected in *C. glabrata* are higher in the ICU settings as compared to non-ICU isolates. Therefore, most ICU patients succumb to the disease because the fungus is not diagnosed early enough, leading to poor prognosis (Niimi *et al.,*2010;Nakajima et al.,2020). The alteration is resistance can therefore be explained by the excessive reliance on antifungal agents in preventive measures that have also caused the resistance. Another feature of the *C. glabrata* is its low inborn predisposition to fluconazole. Thus, in the past few years there has been continued identification of isolates with true resistance to fluconazole. This has led to echinocandins and other azoles become popular despite, this has lead to the emergence of strains resistant to some of these antifungal agents. Thus, the multidrug-resistant variants have appeared. Dispersion in antifungal resistance in *C. glabrata* by the geographical area is evident hence the need to conduct further research on the antifungal susceptibility in localized and regional levels (Martinez-Herrera *et al.,*2020; Frías-De-León *et al.,*2021). Alobaid and Khan (2019) identified that 46 out of 72 *C. glabrat* strains in second-level hospitals in Kuwait, including the intensive care unit, were resistant with a MIC of 64 µg/mL and the remaining 26 were dose dependently susceptible with a MIC of 32µg/mL only. The fungus *C. dubliniensis* exhibited heteroresistant to the antifungal fluconazole but did not show it towards the algal extract. Thus, *C. dubliniensis* was heteroresistant to azoles, mainly due to the development of azole-resistant or, heteroresistant mutations capable of neutralising the effectiveness of azoles. Hetero-resistance is the ability of a subset of microbes within a microbiological population to present variable levels of antibiotic resistance. Thus, specific clones may exhibit resistance while others may display susceptibility. When exposed to antifungal medicines, an adaptive stress mechanism can augment fungi's survival capacity (Scott *et al.,*2023; Huang *et al.,*2024). Ayadi *et al*. (2020) conducted a study in which they examined a phenomenon known as the Trailing phenomenon, which is the incomplete inhibition of fungi when exposed to a dose higher than the minimum inhibitory concentration (MIC). This phenomenon has been observed in the fungus. *C.dubliniensis* is resistant to the antifungal Echinocandin but not to fluconazole.

5.CONCLUSION

The study isolated and analysed Iraq's green alga *Desmodesmus subspicatus*, revealing significant morphological diversity. The methanolic extract showed high efficacy against some multidrug-resistant *Candida* species isolated from the intensive care unit, including *C. glabrata, C. dubliniensis*, and *C. albicans*.

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