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Isolating and identifying yeasts from immunosuppressed patients and testing the effectiveness of the alcoholic and aqueous extract of the fungus Cladosporium cucumerenium on these yeasts.

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Abstract

The study aimed to isolate and diagnose yeasts from patients with cancer, kidney failure, and pulmonary tuberculosis, and to detect the inhibitory effectiveness of the Cladosporium cucumerenium fungus on yeasts that were isolated during the time period from September 2022 to May 2023. It indicated that there were seven types of yeast that were isolated and identified using the ink stain. Indian differentiation media, belonging to the genus Cryptococcus neofrmuns, emerged with three species, C. albidus, C. laurentii, and in the genus Candidiasis with three species, Cadida albicans, C. tropicalis, and C. guilliermondii, and the genus Trichosporon asahi appeared. All species were resistant to the antibiotic amphotericin B. Cryptococcus neofrmuns is less resistant to fungal antibioticsThe highest inhibitory activity was recorded at 100% concentration against Trichosporon asahi, Candida guilliermondi and Cryptococcus neoformuns with an average inhibition diameter of 8, 9 and 5 mm, respectively. The lowest ac-

tivity was recorded against C.albicans and Cryptococcus laurentii with an average inhibition diameter of 1.3 and 0.1 mm, respectively. While the concentration of 50% recorded the highest inhibitory activity against Trichosporon yeast, Candida guiilliermondii, and Cryptococcus neoformuns, with an average inhibition diameter of 0.9, 5.6, and 4.3 mm, respectively. The lowest activity was recorded against the yeast C.albicans and Cryptococcus laurentii with an average diameter of inhibition of 0.1 and 1.7 mm. The inhibitory effect of the alcoholic extract on yeasts was recorded, as the 100% concentration gave the highest inhibitory activity against C.guilliermondii and Trichosporon asahii with an average inhibition diameter of 10.3 and 10.2 mm, respectively. The lowest percentage of inhibition was recorded against C. albicans and Cryptococcus laurentii yeast, where the average diameter of inhibition was 7.1 and 6.1 mm. While the concentration of 50% gave the highest activity against C.guilliermondii and Trichosporon asahii with an average diameter of inhibition of 6.5 and 7.1 mm, respectively, while C.albicans yeast and Cryptococcus laurenti recorded 3.0 and 2.7 mm. The inhibitory activity is due to the alcoholic extract of the C. cucumerenium fungus, which contains secondary compounds of alkaloids, flavonoids and terpenes that have inhibitory activity on microorganisms such as yeasts and bacteria.

Introduction

Fungi are eukaryotic, heterotrophic organisms whose cell wall contains chitin and glucans and have their own genetic characteristics. Fungi are divided according to their shape into unicellular fungi, which are yeasts, and multicellular fungi, which are molds[1] . The reason for causing infection with Candida, Cryptococcus, and other pathogenic fungi is due to causing candidiasis because they possess several virulence factors that make them able to resist the conditions in which they live, as they have the ability to withstand high temperatures 37 in addition to the possibility of changing their form from the yeast form. To the filamentous form, which is known

as dimorphism Di, and the production of enzymes that degrade the blood and the tissue it infects, its attachment to and penetration of cell membranes, and other factors [2]. Cryptococcus yeast, which causes this disease, is a widespread opportunistic fungus in the world that infects humans and most animals [3], Bacterococcal infections have evolved significantly from a single case of infection recorded in 1895 AD to one million cases of infection annually around the world at the present time, as they now take up a large portion of fatal fungal infections that often do not respond to treatment and lead to death. Molecular studies of these yeasts have developed significantly. It is significant, as more than 100 genetic sites associated with the virulence of these yeasts were studied [4] .C.neoformans has a strong tendency to infect the central nervous system more than other sites in the body, causing meningitis, which is the most common form in the case of yeast spread from The main site of infection is the lung [5] . While skin injury is less than in the first case [6] .Cryptococcemia infection of the bloodstream is very dangerous because of the yeast's ability to easily reach other organs of the body [7] .

Endophytes are beneficial microorganisms that all plants possess and live within plant tissues without causing any visible sign of infection or disease. Among all microbial endophytes, endophytic actinomycetes, bacteria, and fungi have received great attention due to their many beneficial compounds with The agricultural importance it provides 8 .Among these endophytic microorganisms, endophytic fungi have attracted much research interest because they not only provide new sources of cytotoxic compounds, but also anticancer [9] and antibacterial substances . In recent years, numerous researches on endophytic fungi have revealed their biodiversity, wide ecological distribution, and numerous interactions with host plants and other microbes in the symbiotic chain [10] .

As a result of the progress that has occurred in recent years in the medical field and the use of modern technologies in treatment, this has led to the

survival of many infected people who were suffering from serious diseases, but at the same time the infection has increased, as it has been observed that an increase in the rate of infection with opportunistic fungi such as (Aspergillus, Candida, and Cryptococcus), which It is one of the most systemic antibiotic-resistant fungi [11]

Materials & Methods

(60) samples were collected from patients attending the Cancer and Chest Cancer Center and the Dialysis Center, after the case was diagnosed by the specialist doctor at Baqubah Teaching Hospital in Diyala Governorate, during the period from October 2022 to January 2023. The study included both genders and their ages ranged from (10-50). Year samples included:

1- Sputum specimen

(30) sputum samples were collected from patients suffering from pulmonary tuberculosis and cancer in tightly sealed and sterile plastic cups. They were then brought to the laboratory and cultured on previously prepared plates of Cryptococcus differentiation medium and Saproid agar medium, and direct examination was done using India ink staining.

2- Urine specimen

The number of urine samples reached (30) samples, and they were collected in tightly sealed plastic containers. They were then brought to the laboratory and centrifuged. The sediment was taken and cultured on previously prepared dishes of Cryptococcus differential agar medium and sabroid agar medium. Direct examination was done using the Indian ink staining method.

Methods that are used to diagnose fungi

Direct microscopic examination

A portion of the sample that was grown on culture media was taken and placed on a glass slide. Then it was covered and passed over a flame, while stirring it led to the crystallization of potassium hydroxide. After that, it was examined using a microscope, where it was examined under a power of Cryptococcus Differential Agar SDA and observed the color and shape

of the colonies [12] .

Diagnosis using India ink stain

Diagnosis is made by adding a drop of India ink to a drop of yeast suspension present on a clean glass slide, through which it is examined under a microscope to determine whether a capsule is present or not. This examination is used to distinguish yeast.

C.neoformans differs from other yeasts that cannot form capsules [13] .

Identification of isolates on Cryptococcus Difterentiai Agar

The test was carried out according to the company's instructions. After preparing the medium, it was inoculated with the yeast isolates under study after activating it on the spore agar medium using the planning method. Then the dishes were prepared at a temperature of 37°C for 24-48 hours. The DifferentiaI Cryptococcus medium works as a selective medium for isolating and growing the Cryptococcus Spp yeast. And identifying colonies belonging to Cryptococcus isolates and differentiating between their types according to the shape and color of the colonies [14] .

The fungus Cladosporium cucumerinum

It was obtained ready from Tikrit University, College of Education, Department of Life Sciences, and diagnosed by PCR Cladosporium cucumerinum, ID: MH464423.1

Preparation of Fungi Extracts

Prepare an aqueous extract of Cladosporium cucumerinum

This is done by mixing (10) grams of the sample in (40) cm3 of distilled water, i.e. a ratio of (4:1) weight/volume. The sample was placed in a Blender device in an ice bath and the mixture was stirred using the electric magnetic motor for at least one to two hours until it was completely mixed. The wall of the fungal cells was disintegrated and torn, then the mixture was left to soak in the refrigerator for 24 hours, then the mixture was filtered using several layers of gauze and filtered again through a funnel using Whatman No1 filter papers and using a vacuum device (Vaccum) in order to get rid of the remaining fibers. And the impurities and non-crushed parts.

Thus, the raw aqueous extract was prepared, which was diluted using an Air Diyes device. Then the sample was placed after drying in an airtight container with tight covers and in conditions free of moisture. The extract was then preserved by freezing until it was used for the current study [15].

Preparation of alcoholic extracts

Use the organic solvent hexan solvent

The method of Riose and his group (1987), modified from the researcher's basic method, was adopted [16] in preparing alcoholic extracts by crushing (10) grams of the fungal sample in (100) cm3 of the organic solvent Hoxan with a concentration of (95%) inside an ice water bath, then filtering the mixture well with a Stirrer device. It was left in the refrigerator for (24) hours, then filtered. The mixture was then filtered using several layers of gauze. In order to get rid of the organic solvent, the mixture was placed in the rotary evaporator device, which works on evaporation under rarefied pressure and at a temperature not exceeding 40%. After the solvent evaporated From the mixture, a layer of extract was formed and dried by cooling under vacuum pressure in a lyophilization device to preserve the active components of the extract. The extract was then preserved by freezing until it is used in the study [15].

Results

The results of the initial isolation of the studied samples showed the presence of seven species belonging to different genera of yeast. Of the total number of isolates, there were 60 isolates, three species, and they occupied sp. Candida ranked first in the total, while Cryptococcus sp. In second place were three types out of the total number, while in third place was the type Trichosporon asahii, as shown in Table (1)

Table (1) Numbers and percentages of yeast species isolated from patient samples under study

Type of fungus	Types of samples		
	Urain	Sputum	
Cadida albicans	5	6	11
C. tropical	6	4	10
C. guilliermondii	5	2	7
Trichosporom asahii	4	2	6
Cryptococcus neoformuns	5	7	12
C. laurentii	4	5	9
C. albidus	1	4	5
the total	30	30	60

Phenotypic and microscopic diagnosis of isolated yeasts

The isolates were diagnosed based on phenotypic characteristics as stated in [12] , and based on cultural characteristics and microscopic examination as stated in [17]

1- Candida spp.

Candida spp species appeared by growing them on PDA culture medium at 37°C for 48 hours, as shown in Figure (1). The isolates growing on the medium appeared in the form of white to cream-colored, circular colonies, and this is consistent with the study of [18] , as the examination showed. Microscopy of isolates belonging to the genus Candida spp. After staining with methyl blue dye, they are spherical to oval or elongated and of varying size, as shown in the figure.

Figure (1): No. (a) showing the phenotypic and microscopic diagnosis of the genus Cadida albicans

Figure (b) shows the phenotypic and microscopic diagnosis of the genus C. tropical

Number (c) shows the phenotypic and microscopic diagnosis of the genus C. guilliermondii

2- Cryptococcus spp

Cryptococcus yeast was initially diagnosed based on the phenotypic characteristics that grew on cyanobacterial medium at 37°C in the form of circular, oval or cream-colored, mucous colonies, as shown in the figure. These results agreed with [19],[20]. This study gave the characteristics of Cryptococcus spp yeast, as it was able to grow on agar medium and gave bright white or transparent colonies with a mucous consistency, and this is the result I agreed and this is due to its possession of a polysaccharide capsule, as the diagnosis was based on the characteristics of those colonies, such as size, shape, and color, with [21] and his group, type, and texture. Microscopic examination of slides prepared from yeast colonies using India ink showed that Cryptococcus spp. yeast. Spherical to oval in shape surrounded by a transparent halo due to its possession of a polysaccharide capsule, which is the most important diagnostic characteristic of this yeast, as all isolates under study showed that it possessed a capsule. The size varied from one type to another and from one medium to another. Most cells have a single bud with a narrow base. Cryptococcus neoformans yeast was distinguished by its Due to their small size and small number of budded cells compared to the yeasts of C. albidus and C. laurentii, figs are distinguished by being larger in size and most of their cells appear to be budded under the microscope, and this study agreed with the study of [22].

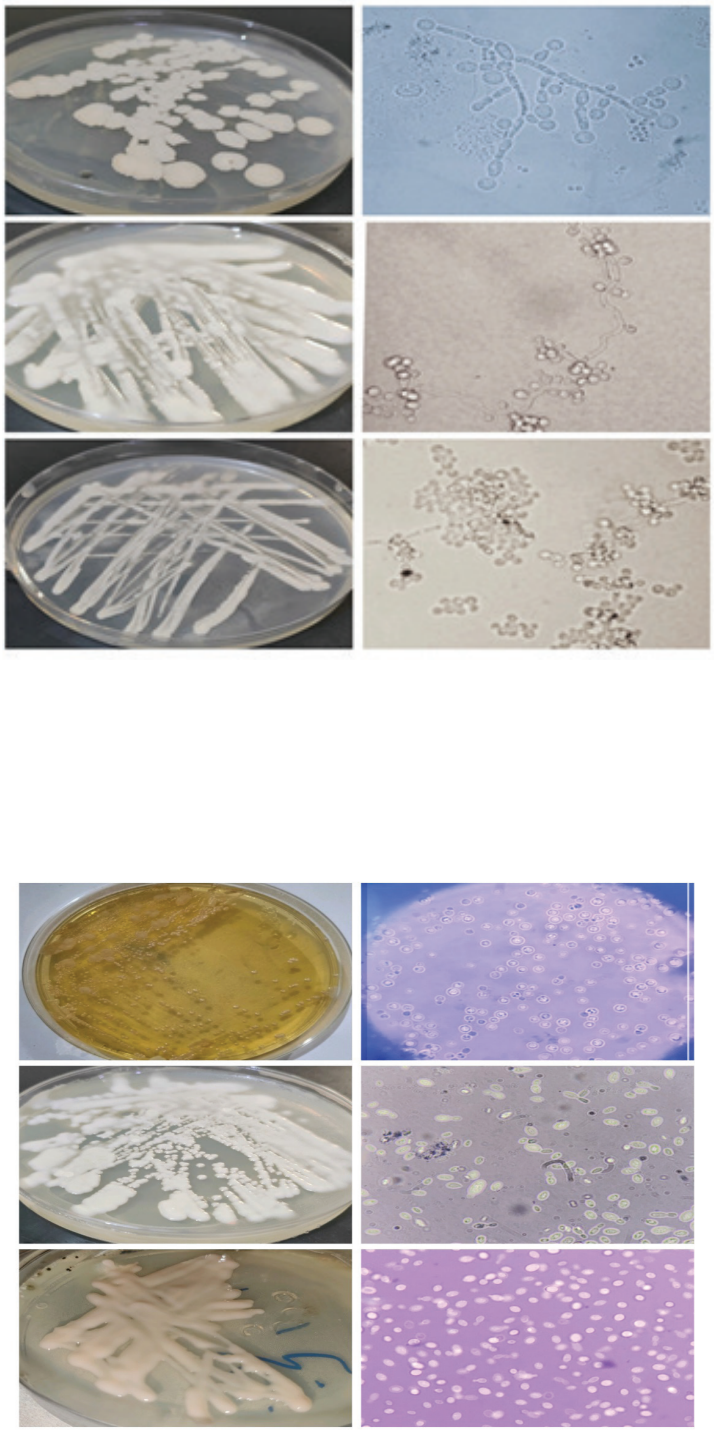

Figure (2): No. (a) showing the phenotypic and microscopic diagnosis of the Cryptococcus neofrmuns

Figure (b) shows the phenotypic and microscopic diagnosis of the C. albidus

Number (c) shows the phenotypic diagnosis of C.laurentii

3- Trichosporon asahii

Trichosporon asahii yeast was identified based on its phenotypic characteristics, as it was able to grow on PDA culture medium, and the colonies were white in color, dry in texture, and rose-like in shape, as shown in the **Figure** (3).

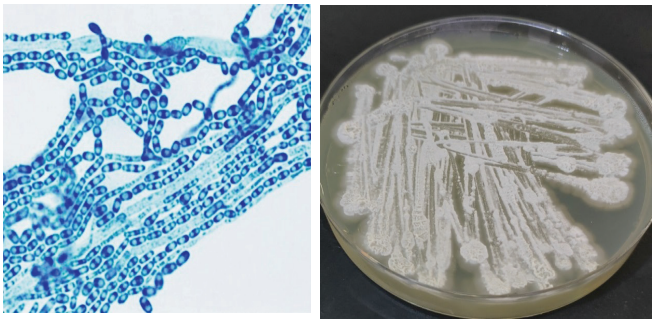

Figure (3): Phenotypic and microscopic diagnosis of Trichosporon asahii yeast

4 - Diagnosis of isolates using Cryptococcus Differential medium

Some isolates were able to grow on Cryptococcus spp. It gave light blue colonies of the type Cryptococcus neoformans, and colonies of a mucusy brown color that characterizes the type Cryptococcus gattii [23] , as in **Figure** (4), and this agrees with the findings of the researcher [18]

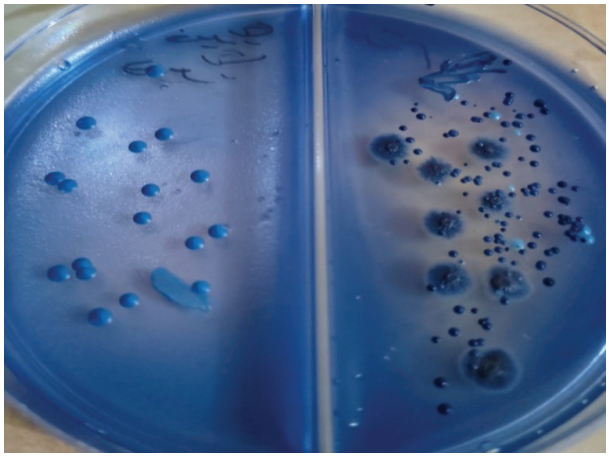

Figure (4): Growth of Cryptococcus neoformans yeast on Cryptococcus differentiation medium

Relationship between Cryptococcus yeast and different parameters

1- The relationship between yeast types and the sex of the patient

The results showed, as shown in Table (2), that there is a difference in the type of yeast for males and females, as C.neoformans' yeast was higher in males (4), followed by C.albidus (2) and C.laurentii (1) in male samples, and this study agreed with [22], studies have indicated that physiological and anatomical differences in both males and females have a direct relationship to the occurrence and recurrence of infection and that the immune response in males is less effective in controlling the infection, as it was found that there are phenotypic differences between the strains isolated from females and males. Of females, it was found that they need double the time to produce (GXM) Glucoronoxlomannan Capsular. It was also found that the macrophages cells in females are more efficient in devouring Cryptococcus yeasts than in males, as the rate of death of macrophages cells increases after devouring the yeasts due to the weight placed on them by those yeasts due to the presence of Virulence factors and metabolic products of yeast, and these are among the reasons that lead to a high incidence of infections in males [24], as is clear in Table (2).

Table (2): Number of isolates of Cryptoc occus spp yeast by genus

the total	Females	Male	
Number of isolates	Number of isolates	Number of isolates	Yeast type
7	3	4	C.neofor-'mans
3	1	2	C.albidus
1	1	1	C.laurentii'
12	5	7	the total

2-The relationship between the type of infection and yeast.

The results showed a high rate of infections in patients with cancer and kidney failure, respectively, as 30 isolates were isolated from these patients out of a total number of isolates after their infections developed into other diseases, where (3) isolates were isolated from the yeast Cryptococcus sp.

From urine samples from those suffering from symptoms of nephritis and urinary tract infection, this is consistent with the findings of [22] in isolating this Cryptococcus yeast from cases of urinary tract infections and chronic kidney failure. The yeast was also isolated from respiratory infections in These patients, as well as those in whom the disease developed and developed meningitis, are because these patients suffer from a weakened immune system due to their use of chemotherapy. As for patients who suffer from symptoms of pulmonary tuberculosis, two isolates were isolated from their sputum samples, and it was found that they were infected with these yeasts and caused them. Symptoms similar to the symptoms of pulmonary tuberculosis. As is known, the way these yeasts enter the body is through inhalation of the yeasts or their spores and cause symptoms similar to the symptoms of pulmonary tuberculosis resulting from infection with tuberculosis bacilli. The infection may remain latent and the patient does not show symptoms of the disease as long as the infected person has an immune system. Healthy, but it becomes active and spreads to the rest of the body after the infected person's immunity weakens [25]

Table (3): Number of isolates of Cryptoc occus spp yeast in terms of infections

Number of isolates	C.laurentii	C.albidus	.C neoformns	Types Typeof injury
14	3	4	7	Cancer
7	1	2	4	Pulmonary tuberculosis
9	2	2	5	Kidney failure
30	6	8	16	the total

Antibiotic susceptibility testing of pathogens.

1- Testing the sensitivity of yeasts to antifungals

Antifungal susceptibility to amphotericin B was tested against some yeast species from different clinical cases, namely Trichosporon asahii, Candida

albicans, Candida guiilliermondii, and Cryptococcus spp. It was noted that all species were resistant to the antifungal amphotericin B, while Cryptococcus neoformous yeast was less sensitive to the antibiotic, as shown in the pictures (5).

Table (4): The effect of antifungals on the yeasts under study

Antifungals		Type of fungus
(amphoterin B) 10	amphotricin B KT)50	
R	R	C.albicans
R	R	C.guilliermondii
R	S	Trichosporon asahii
R	R	Cryptococcus laurentii
R	S	Cryptococcus neoformuns

• R / resistant • S / Sensitive

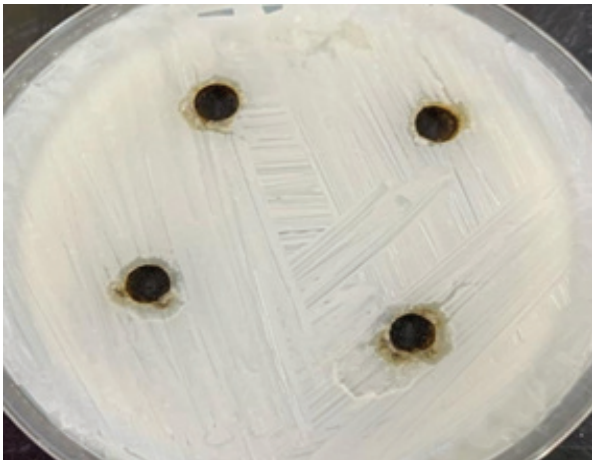

Figure (5) shows the sensitivity of C. albicans and C. neoformous and Trichosporon asahi to the anti- Amphotericin B

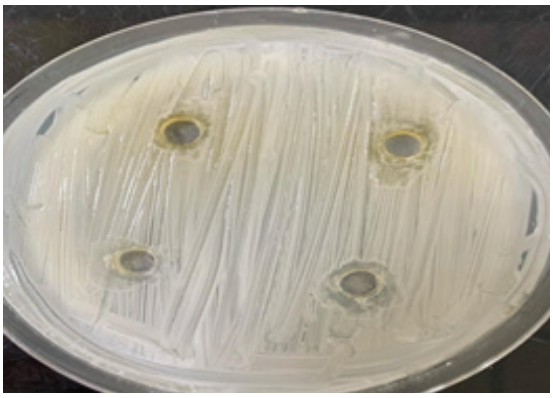

Figure (6) shows the sensitivity of the fungus C.guilliermondii and Cryptococcus laurentii to the antibiotic Amphotericin B

Table (5) shows the inhibitory effect of the aqueous extract on yeasts, where the concentration of 100% gave the highest inhibitory effectiveness, as the highest activity was recorded against C.guilliermondii, Cryptococcus laurentii, and Trichosporon.Asahii yeast with an average diameter and the inhibition percentage reached 8, 9, and 5 mm. Respectively, the lowest effectiveness was recorded. Against C.albicans yeast with an average inhibition diameter of 1.3 mm. While the concentration of 50% was less effective than inhibition.

Table (5) shows the sensitivity of yeasts to the aqueous extract of the fungus

Average type of fungi	Concentration of the aqueous extract		Isolation number	Types of fungi
		50%	**100%**	
0.5	0.0	1.0	**1**	Candida
	0.0	0.0	0.0	Albicans
	1.8	0.5	3.0	**2**
				3
	0.1	**1.3**	Average concentration	

5.5	**4.0**	**7.0**	**1**	Cryptococcus Neoformuns
6.5	**5.0**	**8.0**	**2**	
6.5	**4.0**	**9.0**	**3**	
	4.3	**8**		Average concentration
7.4	6.6	8.2	**1**	Candida Guilliermondii **2** **3**
	5.8	2.2	9.5	
	9.0	8.1	10.0	
	5.6	**9**	Average concentration	
4.2	1.5	6.8	**1**	Trichosporon Asahii **2** **3**
	2.8	1.0	4.5	
	2.2	0.1	4.2	
	0.9	**5.1**	Average concentration	
3.00	2.5	3.5	١	Cryptococcus Laurentii **2** **3**
	1.75	1.5	2.0	
	1.45	1.1	1.8	
	1.7	**2.4**	Average concentration	

• Small letters that are similar horizontally mean that there are no significant differences between them

• Vertically similar capital letters mean that there are no significant differences between them

Table (6) The inhibitory effect of the alcoholic extract on yeasts also appears, as the highest inhibitory activity was recorded at a concentration of 100% against Asahii Trichosporon yeast and Candida guilliermondi, where the average diameter of inhibition was 10.3 and10.2 mm, respectively, and the lowest effectiveness was recorded against C. albicans. and Cryptococcus laurentii yeast. With an average inhibition diameter of 7.1 and 6.1 mm, respectively.

Table (6) shows the inhibitory effect of the alcoholic extract on yeasts

Average type of fungi	Concentration of alcoholic extract		Isolation number	Types of fungi
	50%	ف100%		
4.6	3.0	6.2	١	Candida Albicans
6.5	5.0	8.0	٢	
4.0	1.0	7.0	٣	
	3	7.1	Average concentration	
7.8	5.0	10.5	١	Candida Guilliermondii
7.9	6.4	9.4	٢	
9.6	8.2	11.0	٣	
		6.5	10.3	Average concentration
9.5	8.0	11.0	١	Trichosporon Asahii
6.0	7.3	10.6	٢	
5.0	6.1	9.1	٣	
	7.1	10.2	Average concentration	
5.6	3.6	7.5	١	Cryptococcus Laurentii
4.6	2.8	6.3	٢	
3.1	1.8	4.5	٣	
	2.7	6.1	Average concentration	

Discussion

The results of the current study showed the effectiveness of the extract against Crybtoccosis and some Candida sp. The reason for this effectiveness is that it contains biologically active compounds represented by alkaloids and phenols, as in the study (Jassem, 2022) to estimate the total content of alkaloids and phenols of the extract of the endophytic fungi, as the fungus C. cucumerinum had the highest content of alkaloids and also contained a large percentage of phenols, and it was proven. Musarhad (2023) studied through GC.MS analysis, showing that it contains 19 alkaloid compounds, but in small proportions, and 8 phenolic compounds. In a study, [30]

concluded that the Cladosporium mushroom extract has inhibitory activity against many pathogens, as it was effective against the bacteria Xanthomonas oryzae, Pseudomonas syringae, and the pathogenic fungus Aspergillus flavus. The reason for the antifungal activity is due to it containing active substances such as isocoumarin derivatives. The alcoholic extract of the C. cucumerinum fungus is effective against Candida sp. The reason for this effectiveness is that it contains biologically active compounds represented by alkaloids and phenols. [31] indicated that the Cladosporium genus has an antibacterial effect on the bacteria Escherichia coli, Pseudomonas aeruginosa, Bacillus megaterium, and Staphylococcus aureus, if the active secondary compounds destroy the bacterial cell wall and cause a defect in the cytoplasm, thus causing the formation of holes in the cell wall membranes [32], and this result is consistent with what [33] indicated in their study that the compound that gives the largest percentage of the total area of the compounds diagnosed by (GC-MS) is attributed to biological activity, as well as behenic acid, which is It is a biologically active compound as an antibacterial and antifungal. The inhibitory activity of the fungus C. cucumerinum isolated from the endophytic fungi plant has a clear inhibitory effect on pathogens due to its secretion of a wide range of compounds with high biological activity. Studies have confirmed that a new biologically active compound produced by fungi inside the plant has been identified, including volatile compounds. During the period (2015-2021) from fungi inside the plant belonging to Ascomycetes, Basidiomycetes, and Zygomycetes, all of which confirmed their biological effectiveness as antimicrobials for microbes that cause various diseases and as anti-cancer cells. The most important compounds are alkaloids, benzopyranones, phenols, flavonoids, steroids, and peptides. Peptides, terpenoids, etc Of the products of secondary metabolism, the long-term relationship between the plant and the fungi that symbiote within the plant can lead to the co-evolution and adaptation of both [34] , and this leads to an overlap between genes, which helps in the biosynthesis of active compounds such as common terpenoid com-

pounds.[35,36,37] . [38] stated that a laboratory-grown C. cladosporioides isolate cannot produce secondary metabolites similar to what a plant-symbiotic isolate of the same fungus produces. In other studies, it has been confirmed that endophytic fungi produce various compounds of secondary metabolites similar to what their host plants produce, such as hydrocarbons. The hydrocarbons ester and monoterpenoid also confirmed that Cl. cladosporioides produces a range of monoterpenoids [39 ,40] . This was confirmed by the study [41] that there is a close study between the host and the symbiotic fungi in the production of secondary metabolic compounds that have biological effectiveness through his study to estimate the total content of alkaloids and phenols in orange leaf extract and the filtrates of the fungi commensal with it. These results are not consistent with the results of Jassim's study (2022), which gave the highest inhibitory effectiveness against the yeasts Candida albicans and Candida glabrata, with a diameter of inhibition value of 23 and 20 mm, respectively, as the inhibitory effectiveness is due to the plant containing secondary compounds of alkaloids, flavonoids, and terpenes with antimicrobial activity. [42] .Another study showed the inhibitory activity of the alcoholic extract of orange peel extract against yeasts and molds, including Candida albicans and Aspergillus flavus. [43],. also showed the effectiveness of the alcoholic extract of the orange plant against Candida albicans [44].

Conclusion

It can be concluded from this study that there are a large number of yeasts, such as cryptococci, that cause meningitis and candidiasis, and that the mushroom filtrate extract is able to inhibit these types of yeasts and bacteria because it possesses effective anti-microbial antioxidant compounds, including phenolic compounds, alkaloids, and terpenes.

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