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The compression of the inhibitory effect of root exudates and extraction of Trigonella foenum-graecum L. seeds on Candida auris and molecular detection of ITS gene

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***Abstract*---**A panel of 42 samples of fungal isolates from the ear canal was collected from Salahadin General Hospital in Tikrit city, Iraq. The results showed that only 9 isolates were Candida auris. The root exudates of Trigonella foenum-graecum L. seeds were used for 15 and 25 days, and the exudates were better when used for 25 days of compression of the inhibitory effect of cold aqueous extract. Antifungal susceptibility of candida isolated against 6 antibiotics the Candida isolates showed high resistance to the Clotrimazole, ketoconazole, fluconazole, and lionized as the resistance ratio are 100,100, 77.7, and 88.8% respectively. The ITS region was discovered using a PCR method in Candida isolates ITS segments that encode genotyping of Candida isolates were amplified under optimal circumstances. and the primers were amplified by 700bp.

***Keywords*---**compression, root exudates, extraction, candida auris, trigonella foenum-graecum, ITS.

Introduction

Trigonella foenum-graecum L. is a unique legume crop with many medicinal properties and health benefits. It has been used since ancient times in the pharmaceutical industries, human food, and animal feed. Historically, *Trigonella foenum-graecum* is one of the oldest known medicinal plants, and it is used in Greek, Egyptian, Chinese and Arab medicine (Dhull et al., 2020). It is considered an important plant that is mainly grown in India and many parts of the world. It is unique among legumes because it contains a high percentage of highly soluble dietary fiber. Besides being rich in calcium, iron, and beta-carotene (Tavakoly et al., 2018). *Trigonella foenum-graecum* is considered one of the health-promoting

foods due to the presence of functional compounds such as carotenoids, phenols, free amino acids, and polyunsaturated fatty acids (Nagulapalli Venkata et al. 2017). It is used to treat many diseases such as hypoglycemia hypocholesterolemia and as an antimicrobial, anticancer, and anti-inflammatory (DAS et al., 2018). For ulcers and used as an antifungal and antibacterial. Its roots were also used in the treatment of parasitic infections affecting plants, including *Orobanche crenata* infection, where its roots secrete metabolites that differ in their polarity with the stimulating activity on the germination of seeds of the parasitic plant *Orobanche crenata* (Abbes et al., 2019).

Candida is one of the most common yeasts and is the fourth most common fungal pathogen in many developed countries (Chowdhary et al., 2017). It can cause diseases that are usually superficial and limited to infecting the mouth and mucous membranes, but it can penetrate and enter the bloodstream, and this will lead to inflammation in the internal tissues (Carey et al., 2019) Because it possesses pathogenic factors, it is obligate commensal and its lack of presence is free in environmental media such as soil and water. (Bensasson et al., 2019). *Candida* has historically been a natural flora of the body through its presence in the mouth, gut, and vagina in healthy people, but it can turn into an opportunistic and deadly pathogen (Kumar et al., 2020). The infection caused by *candida* constitutes a heavy burden on public health due to the high death rate, as one of the most common diseases it causes is candidiasis. (Chowdhary et al., 2020). Candidiasis is a broad term that refers to the inflammation of the skin, mucous and internal organs caused by fungi of the genus *Candida*, which can occur at any age and usually occurs in cases of weak acquired immunity from infection with some diseases. *Candida* is closely related to advances in medical technology and is widely recognized as a major cause of morbidity and mortality in hospitals (Kumar et al.2020).

Candida auris has strong virulence factors, including biofilm formation, It not only makes it more infectious but also demonstrates its ability to become resistant to antibiotics by 100 to 1000 times compared to non-biofilm-producing isolates (Sardi et al., 2013). The extracellular material of biomembranes is comprised of proteins and extracellular DNA, biomembranes also have lipid bilayers and polysaccharides, which make it easier for them to stick to any surface. Biofilms make bacteria resistant to antibiotics by using the same processes that code for genes that make bacteria resistant to antibiotics., antibiotic restriction, and even resistance to host immunity (Lee et al., 2020).

The kingdom Fungi is comprised of many different kinds of eukaryotic microorganisms, all of which have relatively small genomes and the ability to grow and reproduce within a short time (Lin et al., 2014). Target functions, pathobiology, virulence, and pathogenicity of medically significant fungus can all be better understood using the genome. Pathogenic fungus can now be genetically manipulated using a variety of genome editing approaches (Nargesi et al., 2021). Genetic manipulation of microorganisms, such as fungi, can be accomplished through the use of genome editing, a versatile engineering tool (Gaj et al., 2016). Therefore, genetic methods have been widely used to understand the pathogenesis of fungal diseases (Perez-Nadales et al., 2014). as well as to understand how antifungal resistance develops (Dudakova et al., 2017)

This study aimed to Isolation of different species of *Candida auris.* from different parts of the human body and its diagnosis using morphological, agricultural, and biochemical tests. The effect of antibiotics and common antifungals. And the molecular diagnosis of fungal species using *ITS*1 and *ITS*4 as well as the effect of *Trigonella foenum-graecum* L. root exudates and extraction method comparing them with the untreated for a different time in reducing the antibiotic resistance of bacteria causing infection compared with antibiotics.

Materials and Methods

Sample of plant collection, extraction, and root exudates

Trigonella foenum-graecum seed was collected from the farm of the faculty of Agriculture, the Baghdad University in October 2020, and the University of Baghdad's Natural History Museum herbarium was responsible for the diagnosis as well as the classification of the plant. To collect root exudates *Trigonella foenum-graecum* using the Hydroponic Culture System (HCS). The seeds were sterilized for two minutes with 1% NaClO, additionally, the seeds were sterilized by being submerged in sterile water and then wrapped in two layers of sterile gauze, and sterilized 500 ml glass container filled with purified water. After 15 and 25 days, the filtrate was collected after the seeds were allowed to germinate on their own. After that, the root exudates went through filtration using a Millipore filter. (Hameed et al. 1973).

Trigonella foenum-graecum seed extract was prepared according to the method described by Anesini and Perez 1993, The cold aqueous extract was prepared by taking 50 g of seed powder in a beaker, adding 500 ml of sterile distilled water, and incubating in a shaking incubator for 24 hours at 37 °C. the aqueous extract was filtered using a Buechner funnel containing a piece of medical gauze and then using filter papers. The filtrate was centrifuged at 2500 rpm for 15 minutes, the filtrate was condensed using a Rotatory evaporator at 45°C, dried at 20-30°C, and kept in a refrigerator until use. Various concentrations (10, 20, and 30%) of aqueous extract were prepared to determine the appropriate concentration for its effect on *Candida auris.* On Mŭeller-Hinton agar, a freshly isolated inoculum was spread, two wells of 6 mm diameter were prepared using a sterile cork – borer, and 50µL of root exudates were filled into the wells and incubated at 37°C for 18-24 hours (Goodfellow et al. 2012).

Fungal collected and diagnosis

In the months of November and December of 2020, a total of 42 samples of ear canal tissue were obtained from the Salahadin General Hospital in Tikrit city, Iraq, in addition, samples were collected from people of all ages. According to MacFaddin, 1980, A series of microscopic and biochemical diagnostic investigations were carried out, and the diagnosis was confirmed by employing an updated version of the Vitek2 Yeast Identification System, Version 8.01 (Biomerieux, France) (Ambaraghassi et al. 2019).

Antifungal Susceptibility test

Antifungal susceptibility of candida was isolated against 6 antibiotics, including (Nystatin, clotrimazole, ketoconazole, Methicillin, fluconazole, and Linezolid) was using the Kirby-Bauer wells method (Abushaheen et al, 2020). the stock solution and final concentration are shown in table 1. Antibiotic powders were obtained from the Samarra Drugs Industry (SDI), Iraq. 1ml of an adequate solvent was used to dissolve a little amount of the antibiotic. After sterilization with a Millipore filter (0.45 m) and storage at (-20 °C), all stock solutions were ready for use.

Table 1
Stock solution and final consternation of antibiotics

Antibiotics	Stock solution mg/ml	Final concentration µg/ml	Solvent
Nystatin	20	50	DMSO
Clotrimazole			DMSO
ketoconazole			DMSO
Methicillin			Absolute alcohol ethanol
fluconazole			Ethanol 70%
Linezolid			Absolute alcohol ethanol

DMSO: Dimethyl sulfoxide

Molecular identification of candida species.

Geneaid™ plant genomic DNA Kit (Geneaid Biotech Ltd., Taiwan) was used to extract genomic DNA from Candida species. The Ribosomal *ITS* region was amplified by PCR with *ITS*1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and *ITS*4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) [25] (White et al., 1990). The thermal cycler PreMix Kit (Bioneer, Korea) was used for PCR (Bio-Rad) reactions following the manufacturer's instructions. The conditions of PCR amplification were as follows: 95°C for 6min; 95°C for the 45s, 59°C for 1min, 72°C for 1min for 35 cycles; and a final extension at 72°C for 5min.

Results and Discussion

9 isolates were obtained from *Candida auris* among the 42 isolates that were isolated from the ear canal of patients in Tikrit city. The colonies appeared in contrasting colors between white and cream with smooth edges and smooth shiny surfaces, and their distinctive yeast-like odor is an important feature in the phenotypic diagnosis. The antifungal sensitivity of all Candida isolates understudy was tested using 6 antifungal solutions, and the results were determined by measuring the growth inhibition zones. The results showed that there was a difference in the resistance of the isolates to the antibiotics, as the Candida isolates showed high resistance to the Clotrimazole, ketoconazole, fluconazole, and Linezolid as the resistance ratio are 100,100, 77.7, and 88.8% respectively, Whereas the Nystatin showed a resistance of 22.2% and the isolates showed a resistance of 11.1% of the Methicillin as shown in table 2.

Table 2
Antifungals' average diameters of inhibition zones for *Candida auris*

Antibiotics	Inhibition zone dimeter (mm)	Resistance ratio (%)
Nystatin	4	22.2
Clotrimazole	14-22	100
Ketoconazole	22-30	100
Methicillin	2	11.1
Fluconazole	20-27	77.7
Linezolid	18-26	88.8

The main purpose of using antibiotics is to obtain mutated isolates that show sensitivity to these antibiotics, and the presence of isolates sensitive to antibiotics may be due to mutation (Deak, 2011) for the resistance of *Candida auris* to anti-bacterial. As for the fungal antibiotics, it showed resistance to all antifungals, except the antifungal nystatin it was sensitive. The ability of nystatin to kill bacteria or inhibit their growth is due to its interaction with cell membranes causing damage to the cell that leads to a change in the selective cell permeability, and due to the ability of nystatin to combine with sterol components in the cell membrane will affect the exudation of important cellular components and cell death (Espinel- Ingroff and others, 1999). Gottlieb et al., 1959 stated that the presence of one of the sterols in the medium prevents the inhibition of fungi by polyenes. And the random and repeated use of this antibiotic leads to the emergence of resistant types of yeasts of Candida spp. Therefore, it is natural for it to differ from one type to another, and it also varies according to the source from which this type was taken (Mizoutani et al., 2012). As for the resistance of the isolates to the rest of the antifungals, it is attributed to the mechanism of overexpression of the Candida drug resistance genes (CDR) genes, as well as the increase in the transporting proteins that will pump the anti-fungal to the outside of the fungal cell. Under sterile conditions, the seeds were grown using the HCS as a medium for the root exudate that was collected, and 15 and 25-day root exudates were collected and the seeds aqueous extraction were used directly after extraction and were inoculating the bacterial isolates on Muller Hinton Agar medium and measuring the width of the inhibitory zone after incubation at 37°C for 24 hours.

Table 3
Inhibition zone diameter of *Trigonella foenum-graecum* seed roots exudates and aqueous extraction

Root exudates		Aqueous extraction		
Days	Inhibition zone dimeter (mm)	Concentration %		
15	6	10	20	30
		Inhibition zone diameter (mm)		
25	15	4	9	12

The antifungal activity of the aqueous extract of *Trigonella foenum-graecum* seed against *Candida auris* was examined. When tested at doses of 10%, 20%, and 30%, the results revealed that both extracts had Antifungal activity against the fungal strain tested. A range of inhibitory zones for the *Candida auris* were shown in Table 3, ranging from 4-12mm. When the antifungal activity of the Root exudates was tested at 15 and 25 days against *Candida auris* results showed that inhibitory zones were 6 and 15 mm, it is noticeable that the diameter of the inhibition zone, when treated with a time of 25 days, is higher than that of the treatment in a shorter time and it gave the highest inhibition zone between both treatments and with the treatment with aqueous extract at all concentrations, and in a study conducted by Al-Hayawi, 2022, it explained that treatment with varying root exudate times gives good results, as long as the time for collecting the exudates is long. The research demonstrated that the root exudates of black seeds were utilized for 10 and 20 days for both magnetic water and nonmagnetic water treatments, Additionally, the exudates were of higher quality after 20 days of treatment with magnetized water.

The allelopathic action of the plant used in this study on root secretions, in most cases, protects the plant and reduces bacterial growth. The secondary metabolites are organic compounds that directly participate in the processes of growth, development, and reproduction of the organism, unlike primary metabolites, which have nothing to do with the survival process. Directly, the absence or loss of secondary metabolic products leads to the direct death of the plant. Rather, its work is related to protecting the plant from insects and animals and increasing its competition with other plants in its environment. The active chemicals found in *Trigonella foenum-graecum* L. seeds are many phenols and thymoquinone compounds that kills germs by reducing infection (Mostafa, 2020). Bacteria and fungi isolates can transfer toxins from the cytoplasm to the plasma membrane and then to the exterior of the cell because they contain efflux pumps. These secreted compounds may work in slowing down or preventing the work of these pumps and depositing the chemicals that the cell lets out and not leaving them outside, ultimately reducing bacterial resistance and killing it (Mosolygó et al., 2019).

The genomic DNA of nine different strains of *Candida auris* was analyzed to determine whether or not they contained the *ITS* regen, and detection of the *ITS* regen gene in *Candida auris* was accomplished with the use of the PCR test., which resulted in bands being visible (700 bp) on a 2% agarose gel (Fig 1).

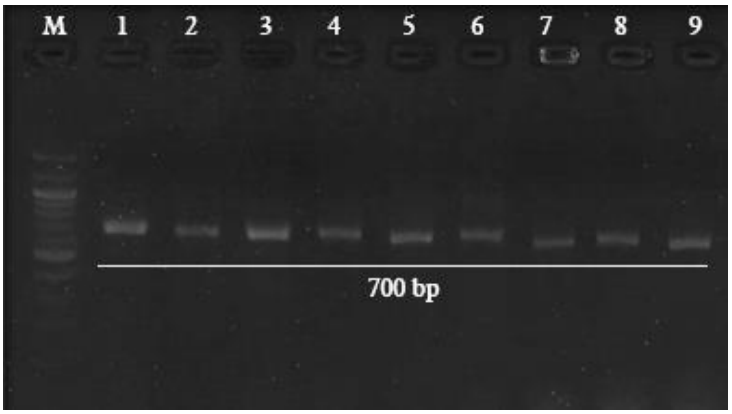

Fig. 1. Electrophoresis of PCR product showing the band size (700 bp) of the *ITS* gene, the product was electrophoresed on 2% agarose at 6 volt/cm^2, DNA ladder (100) lane M., lane 1-9. The *Candida auris* isolates.

Determining the genotypes of the target organisms that determine the degree of genetic similarity or difference at different genetic taxonomic levels, which may be between individuals of one species, strains of a particular species, or different species and races, by adopting PCR products, is what is known as the concept of Genotyping. The PCR technique was utilized in this investigation because of its ability to diagnose one or more genes in the same reaction, as well as the precision and speed with which findings were obtained when detecting antibiotic resistance genes (Forsberg et al., 2019). In fungi, the diagnosis and classification of species and fungal strains of importance have been the focus of scientists' attention. The thinking of finding an advanced taxonomic system or a global code such as DNA barcoding, which represents an easy and quick taxonomic method that uses a short genetic marker within the genome of an organism, is currently used in genetic taxonomic studies *ITS* region, which It is a sequence of nitrogenous bases present on the rDNA of the gene and this region has an important and critical role in the development and function of rDNA (Lockhart et al., 2017)

The results of amplification of the target region of the *ITS*1 and *ITS*4 primers when analyzing the product of gel electrophoresis using agarose at a concentration of 2% showed Fig.1. The appearance of bands of the same molecular sizes and this result show the success of the starter pair in amplifying the target region. The emergence of bundles with similar molecular sizes and this result shows the success of the *ITS* Primaries in amplifying the target region of the rDNA chromosome and this was reflected in the emergence of bundles with similar molecular weights in the size, number, and quality of the nitrogenous bases for the introns and exons of the target region, due to the genetic similarity between the fungal isolates of similar genetic genera (de Groot et al., 2020)

The results of this study contributed to distinguishing between taxonomically close species of the same genus, which had a high diagnostic value, especially for isolates of medical importance, which contributed to the early diagnosis of these fungal infections. When evaluating the efficiency of the genetic sequences of 9 isolates of *Candida auris* and measuring them with the genetic code of the *ITS*

regions, the primers were amplified by 700 bp. This study helped to identify the different taxonomic groups of the fungi in this study.

Conclusions

Candida auris was isolated from Salahadin General Hospital in Tikrit city, Iraq. The gene was identified by the use of a PCR assay for detection, which was based on morphological characteristics and molecular investigations, and the compression of the inhibitory effect of root exudates was treated with water in HCS and extraction of *Trigonella foenum-graecum* L. seeds on *Candida auris* and compared to antibiotics.

Competing Interests

Authors have declared that no competing interests exist

Consent

It is not applicable.

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