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The Efficacy of Antifungal Medications and Plant Extracts Against *Candida albicans* Isolated from Vulvovaginitis Women

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Abstract

Fungus *Candida* spp. causes vulvovaginal candidiasis (VVC) which is a fungal infection of the genital mucosa. This investigation was on the isolation of *Candida albicans* from cervical-vulvovaginal patients in Erbil hospitals, Kurdistan region, Iraq and its identification is done using phenotypic and molecular approaches. Then biofilm production was detected through phenotypic tests such as Congo red agar (CRA) and disclosure virulence genes, agglutinin-like sequence(*ALS1*) and hyphal wall protein (*HWP1*). The susceptibilities of antifungal medications and plant extracts against *C. albicans* isolates were then assessed. This study showed low susceptibilities values to all tested antifungals such as econazole (12mm), miconazole (12mm) and nystatin (14mm) in the disc diffusion method with the exception of ketoconazole (20mm) which had high susceptibilities values. In this research each ethanol pomegranates peel extracts (EPPE) (200mg/ml), aquatic pomegranates peel extracts (APPE) (200mg/ml) and pomegranate molasses (PM) were used. In the agar well diffusion method, *C. albicans* showed low susceptibilities values against (PM) (8mm). However, it showed high susceptibilities values against (EPPE) (18mm) when compared to each of the antifungal agents: econazole, miconazole and nystatin. Results of the study showed that pomegranate peel could be new therapeutic promisingekey ingredient in antifungal medications development. It is an alternate antifungal medication which is useful for *C. albicans* treatment.

Keywords: *Candida albicans*, Biofilm, VVC, Ethanol pomegranates peel extracts, Ketoconazole

فعالية الأدوية المضادة للفطريات والمستخلصات النباتية ضد المبيضات البيض المعزولة عن التهاب الفرج والمهبل لدى النساء

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الخلاصة

تسبب انواع من الفطر *Candida* spp. داء المبيضات الفرجي المهبلي(VVC) ، وهو عدوى فطرية في الغشاء المخاطي للأعضاء التناسلية الانثوية. ان هذا البحث هو حول عزل *Candida albicans*من عنق الرحم والفرج والمهبل لمرضى في مستشفيات أربيل ، اقليم كوردستان ، العراق ، وتم تشخيصها باستخدام الأساليب المظهرية والجزيئية. تم الكشف عن إنتاج الغشاء الحيوي من خلال اختبارات النمط الظاهري مثل Congo red agar (CRA) وكشف لجينات الفوعة *ALS1* و *HWP1*. تم بعد ذلك تقييم حساسية الأدوية المضادة للفطريات والمستخلصات النباتية ضد عزلات *C. albicans*. حيث أظهرت قيم حساسية قليلة لجميع مضادات الفطريات econazole (12mm), miconazole (12mm), n ystatin (14mm)التي تم اختبارها بطريقة disc diffusion method باستثناء k etoconazole(20mm)، حيث كانت له قيم حساسية عالية.في هذا البحث تم استخدام كل من مستخلص قشور الرمان(200mg/ml) (الايثانولي والمائي) ودبس الرمان. بإستخدام طريقة Agar well diffusion ، أظهرت *C. albicans* قيم حساسية قليلة ضد دبس الرمان(8mm)ولكن ت ظهرت قيم حساسيةعالية ضد مستخلصات قشر الرمان بالإيثانول (EPPE)(18mm) عند مقارنتها بكل من المضادات الفطرية:econazole, miconazole, nystatin. قشر الرمان قد يعتبر دواء بديل مضاد للفطريات وهو مفيد لعلاج *C. albicans*. نتيجة لذلك ، يمكن أن يكون لقشر الرمان جانب علاجي جديد كمكون رئيسي في الأدوية المضادة للفطريات.

1. Introduction

Candida albicans is one of the most common fungal infections in human beings [1]. Vulvovaginal candidiasis (VVC) affects almost three-quarters of all women during their reproductive years, and is one of numerous risk factors for cervical intraepithelial neoplasia and cervical cancer. The actual incidence of VVC is difficult to quantify due to the fact that many patients self-treat [2, 3].

Chromogenic agar is a new medium that allows for isolation and then identification of different species of *Candida* as well as detection of polyfungal populations in clinical samples [4]. The capacity to detect mixed yeast cultures is the principal advantage of such chromogenic medium because various species commonly form colonies with distinct colors [5]. As compared to the traditional time-consuming *Candida* species identification methods, the identification of *Candida* species using molecular approaches is quick and accurate [6]. *C. albicans*, an opportunistic pathogenic fungus, produces biofilms as a primary virulence component. However, the effect of antifungal medication on such virulence characteristics has not been well studied. The biofilm formation as well as synthesis of secreted hydrolases may be hampered by typical antifungal drugs [7]. Susceptibility testing of fungi against routinely used antifungal medicines is an important part of the treatment of individuals with fungal infections. In recent decades, antifungal susceptibility testing (AFST) has advanced to the point where it is now standardized and is available in commercial manual/automated phenotypic approaches [8]. The development of alternative antifungal medications is necessitated by the evolution of resistance to the most commonly used antifungal treatments which is also attributable to biofilm formation [9].

Pomegranate is an old fruit that has remained relatively unchanged throughout human history. Punicaceae is the family name for this plant. It is a crucial horticulture fruit in the Mediterranean environment. Saccharides, polyphenols and essential minerals are abundant in the edible section of the fruit [10]. Pomegranate (*Punica granatum*) has been used to cure a variety of ailments and is a rich source of phenolic compounds, particularly hydrolyzable tannins which has strong antioxidant activity. It is typically ingested fresh or in beverages [10, 11].

The aim of this study was to isolate *C. albicans* from cervical-vulvovaginal patients and then identify those using both phenotypic and molecular methods. Evaluation of the biological activity of a plant extracts compared with antifungal agents against *Candida albicans* was done *in vitro*.

2. Materials and Methods

2.1. Plant Material Extraction and Preparation:

Pomegranate fruits were purchased and collected from several markets in Erbil. Pomegranate peel and seed (white and red) were used in the research. Pomegranate molasses is made from the seed by placing pomegranate on a chopping board. Knife was used to remove the top of the pomegranate and to cut the remaining pomegranate lengthways into quarters. The sections were then transferred to a bowl of water. Fingers were used to gently remove the seeds. The peel and white pith were discarded and the seeds were poured into a food processor until crushed which were then strained through a fine sieve into a jug and pressed with back of a spoon to release the juice. Juice was then heated over medium heat and cooked for 20 minutes or until it had reduced and was syrupy. It was then set aside to cool before pouring into an airtight jar to be stored in the fridge. While the peel extracts in both aqueous and alcohol forms were made using water and ethanol. Forty grams of plant powders were weighed and 160 ml of sterile distilled water (sterile DW) was added to make aqueous extract. For alcoholic extracts, 20 g of materials were weighed and then 200 ml of 95% ethanol was added. Each extract was carefully shaken for one hour in a shaker (shaker incubator-4045/gallenkamp-9B/ England) and stored at 4°C for 24 hours and then filtered using Whatman paper before drying in a Petri dish. The powder was then collected and stored in a refrigerator in vials. For each extract, a stock solution was made by combining aqueous plant extract (1gm) with SDW (5 ml) and ethanol plant extract (1gm) with dimethyl sulfoxide (DMSO) (Riedel-DeHaen AG Germany) (5ml), then sterilising it with Millipore filters (0.2µm) [12, 13].

2.2. Samples Collection and Direct Examination

In this investigation, one hundred vaginal swabs were taken by physician from study participants, aged between 18 and 60 years and who had vaginal discharge, irritation, or vulvar pruritus. The samples were collected from October 2019 to August 2020 in Sardam, Dayk and maternity Hospital in Erbil City. All swabs were subsequently sent to mycology laboratory in Salahaddin University- Department of Biologyin the College of Sciences. Each isolate was recovered and cultured aerobically for 24-48 hours at 37°C on sabourauds dextrose agar (SDA)

(Oxoid Ltd., United Kingdom), supplemented with chloramphenicol [14]. The colony was inspected macroscopically and then microscopically by placing a piece of it on a clean glass slide and adding a drop of lactophenol cotton blue. The sample was covered with a fresh cover slip. Light microscope was used to examine the slide, thus enabling a clear view of pseudohyphae as well as blooming oval yeast cells which belonged to different *Candida* species. All isolates were conserved on a slant tube. They were kept in the refrigerator before being inspected to ensure their purity and viability.

2.3. CHROMagar *Candida*

All positive cultures were grown on CHROMagar candida (bioMérieux, France), and incubated at 37°C for 72 hours. *Candida* species identification was preformed based on a colony color [14, 15].

2.4. Molecular Method

DNA Extraction and PCR Amplification

By following the guidelines, *Candida* isolates were cultured for 48 hours on SDA at 37°C and then the DNA was extracted using a genomic DNA extraction kit (Fungi/Yeast Genomic DNA isolation kit. NorgenBiotek/Canada). In two rounds of amplification, multiplex nested PCR was used: First round was done by universal primer (*UNI1* and *UNI2*) to amplify the large target sequence. A total of 25 µl PCR reaction mixture was performed containing 3µl of genomic DNA, 12.5 µl of 2X GoTaq Green Master Mix (Promega/USA) and 1µl was added for each of the forward and reverse primer for both *UNI1* and *UNI2*, forward (*UNI1*, F´5-GTCAAACTTGGTCATTTA-´3), reverse (*UNI2*, R-5´ TTCTTTTCCTCCGCTTATTG-´3)[16, 17], then the mixture was completed by adding 7.5 µl of nuclease free water. The program cycle for *UNI1* and *UNI2* was as follow: initial denaturation of DNA at 94°C for 3 min. The total cycle number 30, each cycle consisted of a denaturation step at 94°C for 30 s, an annealing step at 50°C for 30 s and an extension step at 72 °C for 1 min, with a final extension at 72°C for 10 min then 10µl of PCR product from first round was used as a template for the second round amplification for species level identificationThe master mix preparation for the specific primer was done in 50µl as a total volume, 25µl of 2X GoTaq Green Master Mix (Promega/USA) and 2µl was added for each of the primers,Forward: *Calb*:AGCTGCCGCCAGAGGTCTAA(583/446bp),Forward:*Ckru*:CTGGCCGAGCGAACTAGACT(590/169bp),Forward:Cpar:GTCAACCGATTATTTAATAG(570/370bp), Forward: *Cdub*: CTCAAACCCCTAGGGTTTGG(591/217bp) and Reverse: Clus: TTCGGAGCAACGCCTAACCG [17], then completed by adding 5µl of nuclease free water.

The program file for the above specific primers with minor modification (cycling conditions) were as following: initial denaturation 94°C for 5min, 40 cycles of 15 s at 94°C, 30 s at 55°C, and 45 s at 65°C following a 10-minute period at 94°C for DNA denaturation and enzyme activation. The *ITS* region was amplified via PCR amplification process with a Techne/UK

thermocycler under the following conditions: an initial denaturation cycles at 95°C for 5 minutes, followed by 35 cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute and a last extension for 7 min. at 72°C. The 505bp of PCR products were confirmed by using agarose gel electrophoresis(2%) in 1XTBE buffer and PCR products of *Candida* isolates were sent to Macrogen, South Korea for sequencing [17].

The Sequence Alignment for Targeted Region:

MEGA5 and NCBI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) were used to analyze the sequences. PCR products were sequenced on a 3500 Genetic Analyzer (Applied Biosystems). The sequencing data was then submitted to GenBank (NCBI) for nucleotide sequence accession numbers.

2.5. Antifungal Activity of Plant Extract and Antifungal Drugs Disk

2.5.1. Agar Well Diffusion Method: The antifungal activity of homemade pomegranate molasses and peel extracts (alcoholic and aquatic) was determined using the agar well diffusion method against *C. albicans* isolated. Stock solution was prepared for each of the alcoholic and aquatic extracts. The inoculum of *C. albicans* was prepared using 48-hour-old yeast cultures grown on SDB/2%. They were adjusted to (10^6cell/ml) with a bright line hemocytometer (Hausser Scientific, Horsham, Pa) [18]. Briefly, 0.1 mL suspension of isolates was spread over SDA. A sterile cork borer was used to punch 6 mm diameter wells in the culture medium. Then 100 µL of each concentration of pomegranate molasses, peel extracts and SDW and DMSO (which was regarded as the control for each of Aquatic and ethanol accordingly), was poured into each well until it was completely full. The treated plate was incubated for 24 hours at 37°C. The diameters of inhibition zones were measured in millimeters using a ruler for each antifungal disk and were then compared with control [19, 20].

2.5.2. Disc diffusion method: The experiment was carried out on SDA agar enriched with 8% glucose [21]. *C. albicans* inoculum was prepared as described in the agar well diffusion method. To produce a lawn of the isolate, a sterile cotton wool swab was streaked with inoculum suspension. The disk diffusion method was used to test antifungal susceptibility. All *C. albicans* isolates were evaluated *in vitro* for antifungal drug susceptibility using an antifungal drugs disk (Biorex diagnostics/UK) mainly for econazole 50mg, ketokonazole 50mg, miconazole 50mg and nystatin 100mg. The National Committee for Clinical Laboratory Standards (NCCLS) now recommends this approach as the current reference method.

2.6. Biofilm Formation

2.6.1. Congo red agar method (CRA): Brain heart infusion (BHI) (37g/L), sucrose (50g/L), Congo red stain (0.8g/L) and agar (20g/L) made up the medium. Congo red was made separately as a concentrated aqueous solution that was autoclaved for 15min at 121°C. After the agar had cooled to 55°C, it was added to the solution. The stain formed colored complexes upon reacting

directly with particular polysaccharides in the biofilm. Plates were inoculated and aerobically incubated for 2-3 days at 30°C. Negative test revealed a non-biofilm producer, which is typically pink, but a positive result indicated black colonies with a dry crystalline quality. The experiments were repeated thrice [22].

2.6.2. Detection of ALS1 and HWP1 Genes Linked to the Development of Biofilms: ALS1 and HWP1 were used in the multiplex PCR amplification (Table1). A total of 25 µl of PCR master mix reaction volume was used, including 3 µl of genomic DNA. 12.5 µl of 2X GoTaqGreen Master Mix (Promega/USA) and 1µl was added for each of the forward and reverse primer for both genes and volume was completed with 5.5µl of DNase, RNase free water. The following protocol was used for the PCR: 1 cycle at 94°C for 4 minutes, followed by 35 cycles at 94°C for 30 seconds, 52°C for 1 minute and 72°C for 2 minutes. A 5-minute final extension cycle was done at 72°C. The PCR products were separated on agarose (2%). 318bp and 572bp meant that the amplification was successfully done for *ALS1* and *HWP1* genes (Wealtec, Dolphin-View, USA) [23].

Table 1: Primer sequences of biofilm formation gene

Primers	Sequences (5' →3')	Amplicon size (bp)	Reference
ALS1	Forward´5 GAC TAG TGA ACC AAC AAA TAC CAG A ´3	318	[23]
	Reverse5´CCA GAA GAA ACA GCA GGT GA´3		
HWP1	Forward 5´ ATG ACT CCA GCT GGT TC´3	572	
	Reverse 5´ TAG ATC AAG AAT GCA GC´3		

3. Results And Discussion

Among the 100 vaginal swabs from patients, aged between 18 and 60 years, with cervical-vulvovaginal candidiasis collected in Sardam, Dayk and Maternity Hospital in Erbil City-Iraq, 45% were negative cultures, 8% non-*Candida* species, 46% *C. albicans* isolates and 1% *C. lusitaniae* isolate. All *Candida* isolates phenotypically were identified macroscopically and microscopically (Figure 1). *Candida* species identified on CHROMagar following a 48-hour incubation period at 37°C were based on colony color. *Candida* species arise when this approach is used [15] (Table 2, Figure 2).

According to some of the results presented in resource-constrained situations, the use of CHROMagar *Candida* for rapid identification of *Candida* species directly from clinical specimens could be very valuable in developing appropriate therapeutic strategies and patient management [15]. The results of this study support those of Saeed and Saadallah (2019) who collected samples from patients at hospitals in Duhok Province. Inoculation of these isolates on CHROMagar was performed and *C. albicans* was found to be the most common yeast [24]. Mohsin and Ali (2021) cultured swabs on Sabouraud dextrose agar (SDA) and then identified all isolates macroscopically and microscopically following incubation [25]. Nadeem *et al.* (2010)

demonstrate that CHROMagar *Candida* can easily identify and differentiate between *C. albicans*, *C. tropicalis* and *C. krusei* based on their colonial color and morphology [15]. Hussain *et al.* (2020) used microscopic inspection to identify the *Candida* isolates [26]. Ozcan *et al.*(2010) identified yeasts by conventional methods such as microscopic morphology and chromagar. *C. albicans* and *C. glabrata* were among the 182 isolates. At 72 hours, the efficacy of chromogenic medium for *C. albicans* was 92.9 % [4]. Othman *et al.* (2018) identified *C. albicans* after culturing samples on Chromo agar *Candida* [27].

Figure 1: Microscopic examination showing budding of *Candida albicans* stained by lactophenol cotton blue

Table 2: Colony color of *Candida* isolates on CHROMagar candida medium

Yeast Isolates	Colony Color on ChroMagar
C. albicans	Apple green colonies; consistent
C. lusitaniae	Pink gray purple

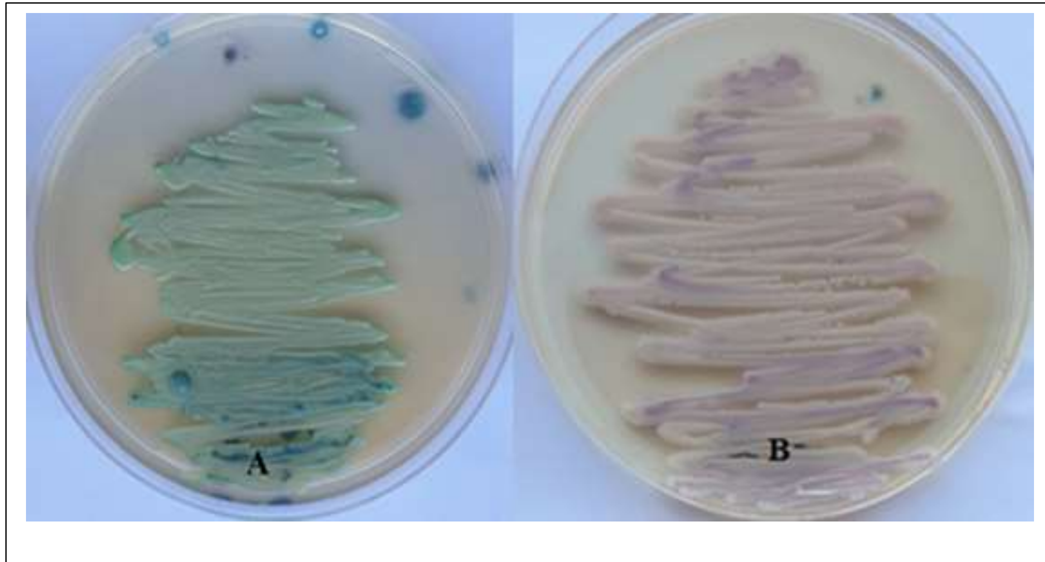

Figure 2: Culture of A. *C. albicans* B. *C. lusitaniae* on CHROMagar candida medium

The results of the molecular identification by using universal and specific primers gene amplification were consistent with the phenotypic analysis which included *C. albicans* and *C. lusitaniae.* Molecular data of specimens from *ITS* nucleotide sequences provided accurate characterization and identification of isolates (Figure 3). The percentage of the alignment nucleotide sequences were arranged between 99.8 and 100%. Four sequence data of *candida albicans* provided GenBank accession numbers for nucleotide sequences (Table 3).

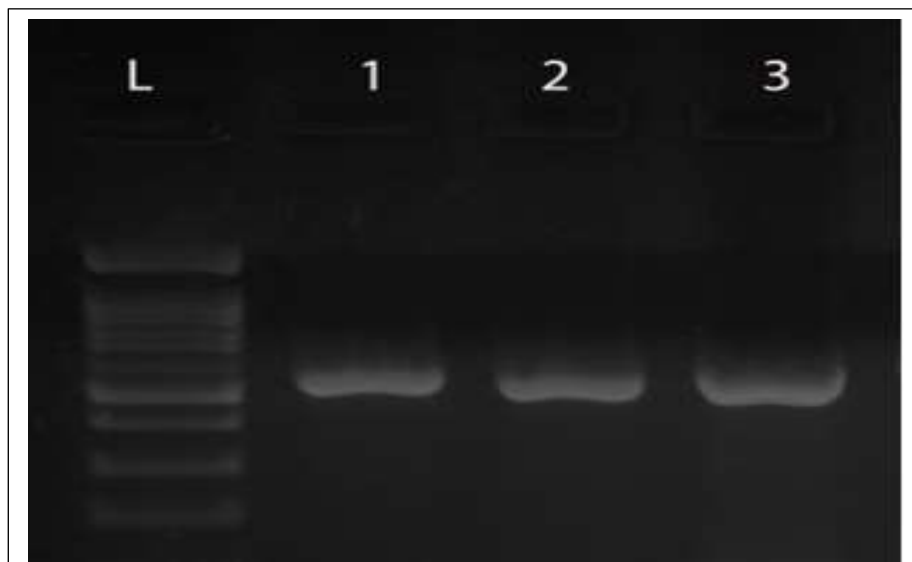

Figure 3: PCR amplification of adjacent segment of the nuclear small subunit rDNA gene. Lane L: DNA ladder 1kb, Lane 1-3: Correspond to fungal ITS region amplification.

Multiplex PCR may be more reliable and time-saving than phenotypic approaches in the identification of *Candida* spp. for diagnostic purposes [28]. Because multiplex-PCR is far more sensitive than culture, it can be recommended as a sensitive and specific assay for *Candida* spp. Identification [29]. Mahmoudi Rad *et al*. (2012) isolated *Candida* and used multiplex PCR to identify the species and show that the most common cause of vulvovaginal candidiasis is *C. albicans* [30]. Carvalho *et al*. (2007) showed that multiplex PCR allows the detection of eight therapeutically important yeasts such as: *C. albicans, C. lusitaniae, C. krusei, C. dubliniensis, C. glabrata,* and *C. parapsilosis* [17].

Table 3: GenBank accession number for four *C. albicans* isolates

C.albicans	GenBank Accession No.
1	MZ165350
2	MZ165351
3	MZ770753
4	MZ770754

Each phenotypic method, using (CRA) and molecular method, utilizing PCR for detection of virulence genes (*ALS1* and *HWP1*), were biofilm producers in the biofilm formation investigation. The development of biofilms was investigated to see if gene presence correlated with phenotype. The findings revealed that both *ALS1* and *HWP1*genes were found in *C. albicans* isolates (Figure 4).

Biofilm was generated by a great number of *C. albicans* isolates [31]. The ability of *Candida* species to generate drug-resistant biofilms is critical to their contribution in human disease. It provides a safe haven for fungi, shielding them from both antimicrobial treatments and host immune system [32]. According to Nobile *et al*. (2006) findings *HWP1* is required for biofilm adherence. It is the first cell surface protein to be linked to biofilm development in *C. albicans in vivo*, making it a promising therapeutic target [33]. By using a multiplex PCR method, İNCİ *et al*. (2013) found that the *ALS1* and *HWP1*genes in *C. albicans* isolates were recovered from a variety of clinical samples [23]. Ali *et al*. (2015) discovered that *C. albicans* isolated from the vaginal area had greater *ALS1* gene, indicating that these isolates were the most virulent [34].

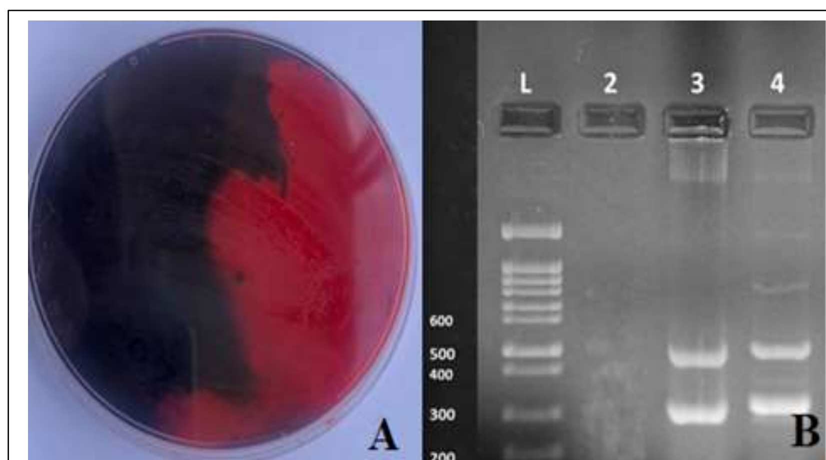

Figure 4: Biofilm formation by A. Congo red agar (CRA) B. Multiplex PCR amplification showing the presence of biofilm genes of *Candida albicans.* Lane 1: Ladder 1kb, Lane 2: Negative control and Lane 3-4: Show coexpression of both *ALS1* and *HWP1*genes.

Antifungal medications, pomegranate peel and molasses were tested against *C. albicans* isolates using agar well and disc diffusion methods. Their growth inhibition zones were also determined. In both procedures the tests were performed on SDA agar supplemented with 8% glucose. In disc diffusion method, xxcept for ketoconazole50 (20mm) which reported high susceptibilities values, *C. albicans* showed low susceptibilities values for all tested azoles antifungals: econazole50 (12mm), miconazole50 (12mm) and nystatin100 (14mm). In agar well diffusion method, *C. albicans* showed high susceptibilities values against ethanol pomegranates peel extracts (EPPE) (18mm) when compared to each of the azoles: econazole, miconazole and nystatin, while the inhibition zone diameter of the others were as follows: aquatic pomegranates peel extract (APPE) (12mm), pomegranate molasses (PM) (8mm). Furthermore, by evaluating DMSO without antifungals, the effect of DMSO on *C. albicans* development was determined, which had no antifungal efficacy. DMSO and SDW were used as a control (Table 4, Figure 5). Pomegranate peel, as an alternate antifungal medication, could possibly help with *C. albicans* infection treatment. As a result, as a significant active component of antifungal medicines, pomegranate peel may have a novel therapeutic potential.

The results of this study were close to those of Mohamed and Thwani (2010) who studied susceptibility test of *Candida* sp. to antifungal drugs and revealed that they were sensitive to miconazole and ketoconazole and were resistant to nystatin [31].While it is in disagreement with Raheem and Ghaima (2021) who found that the highest antibiofilm activity against *candida* species was nystatin [35]. *C. albicans* biofilm development was inhibited by an ethanolic extract of pomegranate. The presence of ellagic acid, bioactive tannin renowned for its antioxidant, anticancer and anti-inflammatory effects, in the ethanolic extract of pomegranate encourages further exploration of the potential of ellagic acid or pomegranate peel powders for the treatment of human illnesses [36].

Table 4: Antifungal susceptibility testing by Agar Well Diffusion and Disc Diffusion method

Antifungal Agent				P. Peel Extract (PPE)				P. Seed (PS)
Diameter of Inhibition Zone (mm)								
ECN	KET	MIC	NYS	APPE	CSDW	EPPE	CDMSO	PM
12mm	20mm	12mm	14mm	12mm	0mm	18mm	0mm	8mm

Note: ECN: Econazole, KET: Ketoconazole, MIC: Miconazole, NYS: Nystatin, APPE: Aquatic pomegranates peel extracts, CSDW: Control Sterilized Distilled Water, EPPE: Ethanol pomegranates peel extracts, CDMSO: Control Dimethyl Sulfoxide and PM: Pomegranate molasses.

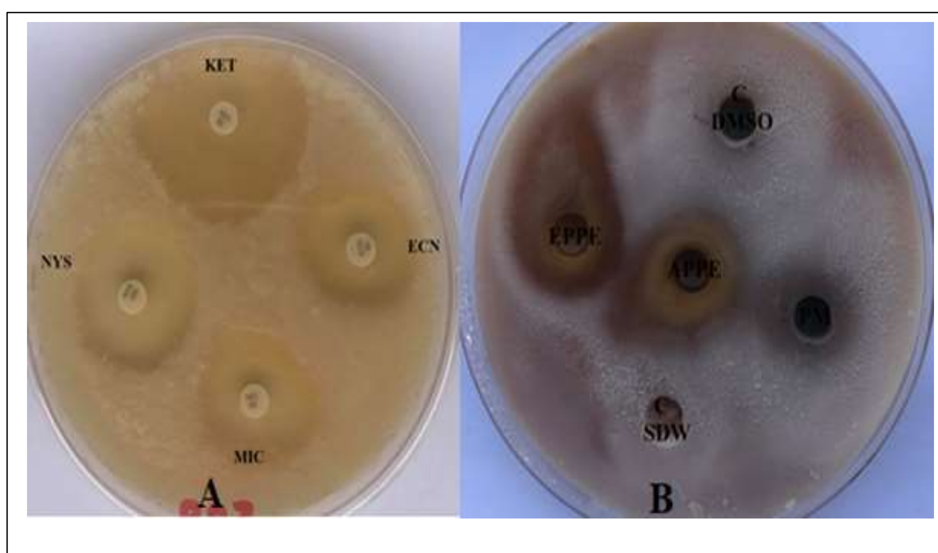

Figure 5: Susceptibility testing for antifungals by: A. Disk diffusion method antifungal azoles: econazol50, ketokonazole50, miconazole50 and nystatin100. B. Agar Well Diffusion: (EPPE), (APPE), (PM), (CDMSO) and (CSDW).

CONCLUSION

These are the first findings to look at the presence of both the ALS1 and HWP1 genes in *C. albicans* strains from Iraqi patients. The current findings point to certain medicines and pomegranate extracts role in controlling yeast infection in women with vulvovaginitis by inhibiting growth of *C. albicans* which is regarded to be the most important virulence factor.

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