ZANCO Journal of Pure and Applied Sciences
The official scientific journal of Salahaddin University-Erbil
https://zancojournals.su.edu.krd/index.php/JPAS

ISSN (print):2218-0230**, ISSN (online):** 2412-3986**, DOI: http://dx.doi.org/10.21271/zjpas**

RESEARCH PAPER

In Vitro Evaluation of Pomegranate (*Punica granatum*) Peel Extract against *Candida krusei* as an alternative agent to antifungal medicines

*Nareen Qadr FaqeAbdulla and Hero M. Ismael
Department of Biology, College of Science, Salahaddin University-Erbil, Kurdistan Region, Iraq

A B S T R A C T:

Abstract:

Vulvovaginal Candidiasis (VVC) is a fungal infection of the genital mucosa caused by *Candida* spp. This is an investigation on the isolation of *Candida krusei* from cervical-VVC patients in Erbil hospitals, Iraqi Kurdistan, and its identification using Chromagar medium. In this study the Susceptibility test of antifungal medications and plant extracts against *C. krusei* isolates were assessed by disc diffusion and agar well diffusion method, respectively. In disc diffusion method *C. krusei* showed various susceptibility ranges against the tested antifungal drugs: Econazole (8mm), Ketoconazole (12mm), Miconazole (10mm) and Nystatin (14mm), while in agar well diffusion method it has been showed higher susceptibilities values against ethanol and aquatic pomegranate peel extract, (15mm) when compared to antifungal drugs. As a result Pomegranate peel is an antifungal alternative that can be used to treat *C. krusei*. According to the LC–MS/MS Technique, both ethanolic and aquatic pomegranate peel extracts were analyzed by Mass Spectrometry and yielded many compounds such as high amount Quinic acid (ethanol: 114.574, water 97.94) mg. analyte/g. extract and Ellagic acid (Ethanol 14.604, Water 48.314) mg/g. extract, while very low amount of Luteolin (E0.005, W0.005) mg/g. extract, Naringenin (E0.007, W0.005) mg/g. extract and Apigenin (E0.003, W0.002) mg/g. extract.

KEY WORDS: *Candida krusei*, Nystatin, Pomegranate, LC–MS/MS Technique, Econazole
DOI: http://dx.doi.org/10.21271/ZJPAS.34.1.7
ZJPAS (2022) , 34(1);69-79 .

1. INTRODUCTION:

Candida is a major Ascomycetous yeast genus with over 150 species, of which more than 20 are clinically important. They live on the skin and mucous membranes of the tracheal, gastrointestinal, and genitourinary systems, among other places (Fotos *et al.*, 1992). *Candida* spp. are the most common fungal pathogens detected in hospitals, producing nosocomial illness (Rodriguez-Leguizamon *et al.*, 2015). *Candida* spp. infection is influenced by a number of characteristics, including age, sex, and the host-pathogen relationship's immunity. They have antifungal resistance to one or more drugs (Othman *et al.*, 2018a).

Chromogenic agar is a novel medium that may be used to identify different Candida species (Ozcan *et al.*, 2010). Because different species typically produce colonies with varying colors, the ability to detect mixed yeast cultures is the primary benefit of such chromogenic media (ChromArt, 2020). Such species must be appropriately identified in order to implement effective management measures and provide appropriate treatment for each species (Rodriguez-Leguizamon *et al.*, 2015).

Antifungal medicines are commonly used to treat VVC, but their effectiveness has been questioned (Qin *et al.*, 2018). Candida species resistance to antifungal drugs could have major consequences for infection control. New antimicrobial medications will be required to prevent and control these illnesses (Bassiri-Jahromi *et al.*, 2015). The evolution of resistance to the most

* **Corresponding Author:**
Nareen Qadr FaqeAbdulla
E-mail:
Article History:
Received: 10/11/2021
Accepted: 05/12/2021
Published: 24/02 /2022

regularly used antifungal therapies necessitates the development of alternative antifungal drugs (Roscetto et al., 2018). Antifungal drugs generated from plants have traditionally been a source of new therapies (Bassiri-Jahromi *et al.*, 2015).

The pomegranate plant belongs to family Punicaceae. The edible part of the fruit is high in saccharides, polyphenols, and vital minerals (Dahham *et al.*, 2010). It is a rich source of antimicrobial activity and phenolic chemicals, notably hydrolyzable tannins, which have strong antioxidant activity, and has been used to treat a range of diseases. It contains active antifungal combinations, reported to have antifungal efficacy against *Candida* spp. (Dahham *et al.*, 2010, Endo *et al.*, 2012, Bassiri-Jahromi *et al.*, 2018).

The aim of the present study was to isolate and identify *Candida* species from Cervical-Vulvovaginal patients in Erbil hospitals then evaluates the in vitro antifungal activity of antifungal medicines: Econazole, Miconazole, Ketoconazole, Nystatin and pomegranate peel extract against *C. krusei*, and to classify and quantify 53 phytochemicals in pomegranate peel extract using a thorough and robust LC–MS/MS (liquid chromatography–tandem mass spectrometry) technique.

2. Material and methods:

2.1. Collection and isolation of samples: Vaginal swabs were taken from patients by obstetric gynecology specialist attending different hospital and gynecology clinic in Erbil city, Iraqi Kurdistan. All swabs were taken from study participants who had vaginal discharge, irritation, or vulvar pruritus then were subsequently sent to mycology laboratory in Department of Biology in the College of Sciences/ Salahaddin University. Each swab was inoculated onto Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol (Oxoid, Basingstoke, UK) and incubated at 37°C for 48hr (Yonis *et al.*, 2019). After incubation, Lactophenol cotton blue stain and microscopical analysis were performed (40X) to identify the yeast. All the yeast isolates were grown on Chromogenic agar (bioMérieux, France), for identification of candida species based on a colony color (Nadeem *et al*., 2010).

2.2. Preparation of the plant extracts for LC–MS/MS and Antifungal activity: Pomegranate fruits were purchased and collected from several markets in Erbil city; the peels were used in this research. Both aqueous and alcohol forms of Pomegranate peel extracts were made using water and ethanol. 40g of peel powder was weighed and mixed with 160 ml of sterile distilled water (SDW) to prepare aqueous extract. For alcoholic extracts, 20 g of materials were weighed, then 200 ml of 95% ethanol was added, Each extract was carefully shaken for an hour in a shaker (shaker incubator-4045/gallenkamp-9B/ England) and stored at 4°C for 24hr, filtered with Whatman paper and dried in a Petri dish, the powder was collected in vials and stored in a refrigerator. 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 sterilizing it with (Millipore filters 0.2µm) (Ríos *et al.*, 1987, Cai *et al.*, 2011). Both ethanol and aquatic pomegranate peel extract powder were sent to Research and Application of Science and Technology Center (DUBTAM), 21280 Dicle University, Diyarbakır, for determination the active compound. It was performed using Shimadzu LC-MS 8040 model triple quadrupole mass spectrometer equipped with an ESI source operating in both positive and negative ionization modes. LC-MS/MS data were collected and processed by Lab Solutions software (Shimadzu, Kyoto, Japan) (Foss *et al.*, 2014, Yilmaz, 2020). **2.3. Method validation studies for LC–MS/MS:** In this study, a comprehensive and robust LC–MS/MS method was developed, optimized, and validated to qualify and quantify 53 fingerprint phytochemicals in plant species. The performance parameters of the devised analytical method were determined using external and internal reference solutions, as well as fortified and non-fortified samples. The parameters related to the validation of LC–MS/MS methods are shown in (Table 1) (Yilmaz, 2020).

2.4. Mass spectrometer and chromatography conditions: A Shimadzu LCMS-8040 tandem mass spectrometer with an electrospray ionization (ESI) source working in both negative and positive ionization modes was used for mass spectrometric detection. Figure 1 shows Mass spectrometer and chromatography conditions (Yilmaz, 2020).

2.5. Antifungal activity of plant extract and antifungal drugs disk

2.5.1. Agar Well Diffusion Method: Antifungal activity of pomegranate peel extracts (ethanol and aquatic) was tested against *C. krusei* isolates. The stock solution was prepared for both extracts. The inoculum of *C. krusei* was prepared using 48hr old

yeast cultures grown on sabouraud dextrose broth (SDB) and they were adjusted to (10^6/ml) with a bright line hemocytometer (Hausser Scientific, Horsham, Pa) (Aboualigalehdari *et al.*, 2016). Briefly, 0.1 mL suspension of isolates was spread over sabouraud dextrose agar (SDA); a sterile corkborer was used to punch 6 mm diameter wells in the culture media. Then, 100µL of each extracts and (SDW and DMSO) as control, was poured into each well until it was completely full. The treated plate was incubated at 37˚C for 24hr. The diameters of zones of inhibition were measured in millimeters using a ruler for each antifungal disk and compared with control (Aibinu et al., 2006, Srinivasan *et al.*, 2001).

2.5.2. Disc diffusion method: The experiment was carried out on SDA. The inoculum of *C. krusei* 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. The isolate was evaluated *in vitro* for antifungal drug susceptibility using an antifungal drugs disk (Biorex diagnostics/UK), mainly for Econazole50mg, Ketokonazole50mg, Miconazole50mg, and Nystatin100mg. The National Committee for Clinical Laboratory Standards (NCCLS) now recommends this approach as the current reference method (Othman *et al.*, 2018a).

3. RESULTS AND DISCUSSION

C. krusei were isolated from Cervical-Vulvovaginal candidiasis patients in various hospitals in Erbil. These isolates identified macro and microscopically, the positive culture (*Candida* sp.) identified on CHROM Agar following 48hr incubation period at 37°C based on a colony color. It appears light pink colonies (Table 2, Figure2).

Candida isolates were subjected for the phenotypic approaches CHROMagar confirmation depending on the color (Liguori *et al.*, 2010). According to some of the results presented, in resource-constrained situations, the use of CHROMagar Candida for rapid identification of Candida species from clinical specimens could be very valuable in developing appropriate therapeutic strategies and patient management (Nadeem *et al*., 2010). Our findings are consistent with those of Habib et al., who used CHROMagar to identify Candida spp. causing vulvovaginal Candidiasis, including *C. albicans, C. krusei, C. tropicalis , C. parapsilosis* and *C. glabrata* (Habib *et al*., 2016). Nadeem *et al*., who demonstrate that CHROMagar Candida can easily identify and differentiate between *C. albicans*, *C. tropicalis* and *C. krusei* based on colonial color and morphology (Nadeem *et al.*, 2010). Mohsin and Ali, who cultured swabs on (SDA), then identified all isolates macroscopically and microscopically following incubation (Mohsin and Ali, 2021). Othman et al., who identifying Candida spp. after culturing samples on Chromogenic agar Candida (Othman *et al*., 2018b).

The antifungal drugs: Econazole, Ketoconazole, Miconazole and Nystatin were tested against *C. krusei* using Disc diffusion method. For testing pomegranate peel extract, Agar well diffusion method is used. The sensitivity test was depending on zone of inhibition and their growth inhibition zones were determined. In both methods, the tests were performed on SDA agar.

In the Disc diffusion method, *C. krusei* showed various susceptibility ranges against antifungal drugs: the high inhibition zone appeared in Nystatin 100mg (14mm), followed by Ketoconazole 50mg (12mm), Miconazole 50mg (10mm) and Econazole50mg (8mm). While in Agar Well Diffusion method, *C. krusei* showed higher susceptibility values against both water and ethanol pomegranates peel extracts, the diameter of inhibition zone was (15mm) (DMSO and SDW used as a control), which was higher than all tested antifungal drugs (Table3, Figure3).

Plants are one of a prefect source of natural effective compounds that have antimicrobial, and other activities(El-Desouky, 2021). Several products have been developed to eliminate or reduce potential pathogenic microorganisms. The continuous use of synthetic products can result in side effects such as vomiting, diarrhea, and the induction of microbial resistance. Pomegranate (*Punica granatum*) peel decoction was tested to assess its antimicrobial activity (Leite *et al.*, 2014). It has a long history as food Medicine and still continues in the evolution. It is act as antioxidant ,antibacterial anticancer, and anti-fungal activities, a gel made from pomegranate peel has a high polyphenolic content demonstrated wound-healing capacity (Shaokat *et al*., 2007).

Our results were in agreement with those of Gandhi *et al*., who showed that in Antifungal susceptibility pattern, candida isolates were more sensitive to Nystatin, compared to that of Clotrimazole, Fluconazole and Ketoconazole (Gandhi *et al*., 2015). While it was in

disagreement with those of Mohamed and Thwani, who studied susceptibility test of *Candida* spp. to antifungal drugs and revealed that they were sensitive to Miconazole and Ketoconazole and were resistant to Nystatin (Mohamed and Thwani, 2010).

Candida sp. 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 (Bakkiyaraj *et al*., 2013). Anibal *et al.,* showed that Ethanolic crude extract of Pomegranate (*Punica granatum*) peel had their activity tested against *Candida* spp. (Anibal *et al*., 2013).

The Ethanolic and Water crude extract of Pomegranate peel were analyzed according to the LC–MS/MS investigation by Mass Spectrometry and yielded many compounds (Table 4, Figure4). They contained high amount of Quinic acid (ethanol: 114.574, water 97.94) mg. analyte/g. extract; and Ellagic acid (Ethanol 14.604, Water 48.314) mg/g, followed by Gallic acid (E4.204, W7.117)mg/g, Astragalin (E0.544, W0.461)mg/g, Fumaric aid (E0.452, W0.38)mg/g, Aconitic acid (E0.369, W0.449)mg/g, Nicotiflorin (E0.324, W0.301)mg/g, Isoquercitrin (E0.163, W0.127)mg/g, Rutin (E0.092, W0.079)mg/g, Cosmosiin (E0.054, W0.035)mg/g, Hesperidin (E0.032, W0.03)mg/g, Protocatechuic acid (E0.029, W0.049)mg/g, Acacetin (E0.01, W0.007)mg/g, while very low amount of Luteolin (E0.005, W0.005)mg/g, Naringenin (E0.007, W0.005)mg/g and Apigenin (E0.003, W0.002)mg/g.

Our result is in agreement with Yilmaz *et al*., who performed phytochemical screening of ethanol and methanol-chloroform extracts of some plant by using the developed and validated LC-MS/MS method. It appeared that Quinic acid, being found predominantly in all extracts studied, was more abundant (Yilmaz *et al*., 2018). Anibal *et al.,* who analyzed Ethanolic crude extract of Pomegranate (*Punica granatum*) peel by Mass Spectrometry and yielded many compounds such as punicalagin and galladydilacton. ellagic acid, quercetin, delphinidin, gallagyldilacton and punicalagin (Anibal *et al*., 2013).

4. Conclusions: This study indicated that *C. krusei* were isolate from cervical vulvovaginal candidiasis patients, its colony is light pink on Chromogenic agar, and it has high susceptibility against both ethanol and aquatic crude extract of pomegranate (*Punica granatum*) peel when compared to antifungal drugs: Econazole, Ketoconazol, Miconazole and Nystatin. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) Technique indicated that Pomegranate peel extract contained high amount of active compound. It may serve as a natural alternative option due to its potency against *C. krusei*, indicating that the pomegranate is a good target for study to obtain a new drug.

Ethics and Consent Statement

The consent of the patient was taken in writing through the term of informed consent after this study is approved by the human ethics committee of the university of salahaddin-Erbil. Inclusion criteria were vaginal swabs from women with cervical-vulvovaginal candidiasis who tended to participate in the study. All procedures performed in this studies involving women participant were in accordance with the rules on patient sampling and fully complied with ethics committee

Acknowledgments

We thank the patient for participating in the study. Our special thanks go to Maternity Teaching Hospital at the gynecology clinic in Erbil city, Iraqi Kurdistan and university of salahaddin-college of science-Biology Department for providing the opportunity to carry out this work.

Conflict of interest

There is no conflict of interest

Table 1: Analytical method validation parameters that belong to the LC-MS/MS method

No	Analytes	RT[a]	M.I. (m/z)[b]	F.I. (m/z)[c]	Ion. mode	Equation	r^{2}[d]	*RSD%*[e] Interday	Intraday	Linearity Range (mg/L)	*LOD*/*LOQ* (µg/L)[f]	Recovery (%) Interday	Intraday	*U*[g]	Gr. No[i]
1	Quinic acid	3.0	190.8	93.0	Neg	*y*=-0.0129989+2.97989×	0.996	0.69	0.51	0.1-5	25.7/33.3	1.0011	1.0083	0.0372	1
2	Fumaric aid	3.9	115.2	40.9	Neg	*y*=-0.0817862+1.03467×	0.995	1.05	1.02	1-50	135.7/167.9	0.9963	1.0016	0.0091	1
3	Aconitic acid	4.0	172.8	129.0	Neg	*y*=-0.7014530+32.9994×	0.971	2.07	0.93	0.1-5	16.4/31.4	0.9968	1.0068	0.0247	1
4	Gallic acid	4.4	168.8	79.0	Neg	*y*=0.0547697+20.8152×	0.999	1.60	0.81	0.1-5	13.2/17.0	1.0010	0.9947	0.0112	1
5	Epigallocatechin	6.7	304.8	219.0	Neg	*y*=-0.00494986+0.0483704×	0.998	1.22	0.73	1-50	237.5/265.9	0.9969	1.0040	0.0184	3
6	Protocatechuic acid	6.8	152.8	108.0	Neg	*y*=0.211373+12.8622×	0.957	1.43	0.76	0.1-5	21.9/38.6	0.9972	1.0055	0.0350	1
7	Catechin	7.4	288.8	203.1	Neg	*y*=-0.00370053+0.431369×	0.999	2.14	1.08	0.2-10	55.0/78.0	1.0024	1.0045	0.0221	3
8	Gentisic acid	8.3	152.8	109.0	Neg	*y*=-0.0238983+12.1494×	0.997	1.81	1.22	0.1-5	18.5/28.2	0.9963	1.0077	0.0167	1
9	Chlorogenic acid	8.4	353.0	85.0	Neg	*y*=0.289983+36.3926×	0.995	2.15	1.52	0.1-5	13.1/17.6	1.0000	1.0023	0.0213	1
10	Protocatechuic aldehyde	8.5	137.2	92.0	Neg	*y*=0.257085+25.4657×	0.996	2.08	0.57	0.1-5	15.4/22.2	1.0002	0.9988	0.0396	1
11	Tannic acid	9.2	182.8	78.0	Neg	*y*=0.0126307+26.9263×	0.999	2.40	1.16	0.05-2.5	15.3/22.7	0.9970	0.9950	0.0190	1
12	Epigallocatechin gallate	9.4	457.0	305.1	Neg	*y*=-0.0380744+1.61233×	0.999	1.30	0.63	0.2-10	61.0/86.0	0.9981	1.0079	0.0147	3
13	1,5-dicaffeoylquinic acid	9.8	515.0	191.0	Neg	*y*=-0.0164044+16.6535×	0.999	2.42	1.48	0.1-5	5.8/9.4	0.9983	0.9997	0.0306	1
14	4-OH Benzoic acid	10.5	137,2	65.0	Neg	*y*=-0.0240747+5.06492×	0.999	1.24	0.97	0.2-10	68.4/88.1	1.0032	1.0068	0.0237	1
15	Epicatechin	11.6	289.0	203.0	Neg	*y*=-0.0172078+0.0833424×	0.996	1.47	0.62	1-50	139.6/161.6	1.0013	1.0012	0.0221	3
16	Vanilic acid	11.8	166.8	108.0	Neg	*y*=-0.0480183+0.779564×	0.999	1.92	0.76	1-50	141.9/164.9	1.0022	0.9998	0.0145	1
17	Caffeic acid	12.1	179.0	134.0	Neg	*y*=0.120319+95.4610×	0.999	1.11	1.25	0.05-2.5	7.7/9.5	1.0015	1.0042	0.0152	1
18	Syringic acid	12.6	196.8	166.9	Neg	*y*=-0.0458599+0.663948×	0.998	1.18	1.09	1-50	82.3/104.5	1.0006	1.0072	0.0129	1
19	Vanillin	13.9	153.1	125.0	Poz	*y*=0.00185898+20.7382×	0.996	1.10	0.85	0.1-5	24.5/30.4	1.0009	0.9967	0.0122	1
20	Syringic aldehyde	14.6	181.0	151.1	Neg	*y*=-0.0128684+7.90153×	0.999	2.51	0.77	0.4-20	19.7/28.0	1.0001	0.9964	0.0215	1
21	Daidzin	15.2	417.1	199.0	Poz	*y*=9.45747+152.338×	0.996	2.25	1.32	0.05-2.5	7.0/9.5	0.9955	1.0017	0.0202	2
22	Epicatechin gallate	15.5	441.0	289.0	Neg	*y*=-0.0142216+1.06768×	0.997	1.63	1.28	0.1-5	19.5/28.5	0.9984	0.9946	0.0229	3
23	Piceid	17.2	391.0	135/106.9	Poz	*y*=0.00772525+25.4181×	0.999	1.94	1.16	0.05-2.5	13.8/17.8	1.0042	0.9979	0.0199	1
24	*p*-Coumaric acid	17.8	163.0	93.0	Neg	*y*=0.0249034+18.5180×	0.999	1.92	1.43	0.1-5	25.9/34.9	1.0049	1.0001	0.0194	1
25	Ferulic acid-D3-IS[h]	18.8	196.2	152.1	Neg	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0.0170	1
26	Ferulic acid	18.8	192.8	149.0	Neg	*y*=-0.0735254+1.34476×	0.999	1.44	0.53	1-50	11.8/15.6	0.9951	0.9976	0.0181	1
27	Sinapic acid	18.9	222.8	193.0	Neg	*y*=-0.0929932+0.836324×	0.999	1.45	0.52	0.2-10	65.2/82.3	1.0031	1.0037	0.0317	1
28	Coumarin	20.9	146.9	103.1	Poz	*y*=0.0633397+136.508×	0.999	2.11	1.54	0.05-2.5	214.2/247.3	0.9950	0.9958	0.0383	1

[a]R.T.: Retention time, [b]MI (*m/z):* Molecular ions of the standard analytes (m/z ratio), [c]FI (*m/z):* Fragment ions [d]r^2: Coefficient of determination, [e]*RSD*: Relative standard deviation, [f]*LOD*/*LOQ* (µg/L): Limit of detection/quantification, [g]*U* (%): percent relative uncertainty at 95% confidence level (k = 2), [h]IS: Internal standard, [i]Gr. No: Represents grouping of internal standards, these numbers indicate which IS stands for which phenolic compound.

Table 1: Analytical method validation parameters that belong to the LC-MS/MS method (Continued)

No	Analytes	RT[a]	M.I. (m/z)[b]	F.I. (m/z)[c]	Ion. mode	Equation	r^{2}[d]	*RSD%*[e] Interday	Intraday	Linearity Range (mg/L)	*LOD*/*LOQ* (µg/L)[f]	Recovery (%) Interday	Intraday	*U*[g]	Gr. No
29	Salicylic acid	21.8	137.2	65.0	Neg	*y*=0.239287+153.659×	0.999	1.48	1.18	0.05-2.5	6.0/8.3	0.9950	0.9998	0.0158	1
30	Cynaroside	23.7	447.0	284.0	Neg	*y*=0.280246+6.13360×	0.997	1.56	1.12	0.05-2.5	12.1/16.0	1.0072	1.0002	0.0366	2
31	Miquelianin	24.1	477.0	150.9	Neg	*y*=-0.00991585+5.50334×	0.999	1.31	0.95	0.1-5	10.6/14.7	0.9934	0.9965	0.0220	2
32	Rutin-D3-IS[h]	25.5	612.2	304.1	Neg	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	2
33	Rutin	25.6	608.9	301.0	Neg	*y*=-0.0771907+2.89868×	0.999	1.38	1.09	0.1-5	15.7/22.7	0.9977	1.0033	0.0247	2
34	isoquercitrin	25.6	463.0	271.0	Neg	*y*=-0.111120+4.10546×	0.998	2.13	0.78	0.1-5	8.7/13.5	1.0057	0.9963	0.0220	2
35	Hesperidin	25.8	611.2	449.0	Poz	*y*=0.139055+13.2785×	0.999	1.84	1.35	0.1-5	19.0/26.0	0.9967	1.0043	0.0335	2
36	*o*-Coumaric acid	26.1	162.8	93.0	Neg	*y*=0.00837193+11.2147×	0.999	2.11	1.46	0.1-5	31.8/40.4	1.0044	0.9986	0.0147	1
37	Genistin	26.3	431.0	239.0	Neg	*y*=1.65808+7.57459×	0.991	2.01	1.28	0.1-5	14.9/21.7	1.0062	1.0047	0.0083	2
38	Rosmarinic acid	26.6	359.0	197.0	Neg	*y*=-0.0117238+8.04377×	0.999	1.24	0.86	0.1-5	16.2/21.2	1.0056	1.0002	0.0130	1
39	Ellagic acid	27.6	301.0	284.0	Neg	*y*=0.00877034+0.663741×	0.999	1.57	1.23	0.4-20	56.9/71.0	1.0005	1.0048	0.0364	1
40	Cosmosiin	28.2	431.0	269.0	Neg	*y*=-0.708662+8.62498×	0.998	1.65	1.30	0.1-5	6.3/9.2	0.9940	0.9973	0.0083	2
41	Quercitrin	29.8	447.0	301.0	Neg	*y*=-0.00153274+3.20368×	0.999	2.24	1.16	0.1-5	4.8/6.4	0.9960	0.9978	0.0268	2
42	Astragalin	30.4	447.0	255.0	Neg	*y*=0.00825333+3.51189×	0.999	2.08	1.72	0.1-5	6.6/8.2	0.9968	0.9957	0.0114	2
43	Nicotiflorin	30.6	592.9	255.0/284.0	Neg	*y*=0.00499333+2.62351×	0.999	1.48	1.23	0.05-2.5	11.9/16.7	0.9954	1.0044	0.0108	2
44	Fisetin	30.6	285.0	163.0	Neg	*y*=0.0365705+8.09472×	0.999	1.75	1.19	0.1-5	10.1/12.7	0.9980	1.0042	0.0231	3
45	Daidzein	34.0	253.0	223.0	Neg	*y*=-0.0329252+6.23004×	0.999	2.18	1.73	0.1-5	9.8/11.6	0.9926	0.9963	0.0370	3
46	Quercetin-D3-IS[h]	35.6	304.0	275.9	Neg	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	3
47	Quercetin	35.7	301.0	272.9	Neg	*y*=+0.00597342+3.39417×	0.999	1.89	1.38	0.1-5	15.5/19.0	0.9967	0.9971	0.0175	3
48	Naringenin	35.9	270.9	119.0	Neg	*y*=-0.00393403+14.6424×	0.999	2.34	1.69	0.1-5	2.6/3.9	1.0062	1.0020	0.0392	3
49	Hesperetin	36.7	301.0	136.0/286.0	Neg	*y*=+0.0442350+6.07160×	0.999	2.47	2.13	0.1-5	7.1/9.1	0.9998	0.9963	0.0321	3
50	Luteolin	36.7	284.8	151.0/175.0	Neg	*y*=-0.0541723+30.7422×	0.999	1.67	1.28	0.05-2.5	2.6/4.1	0.9952	1.0029	0.0313	3
51	Genistein	36.9	269.0	135.0	Neg	*y*=-0.00507501+12.1933×	0.999	1.48	1.19	0.05-2.5	3.7/5.3	1.0069	1.0012	0.0337	3
52	Kaempferol	37.9	285.0	239.0	Neg	*y*=-0.00459557+3.13754×	0.999	1.49	1.26	0.05-2.5	10.2/15.4	0.9992	0.9990	0.0212	3
53	Apigenin	38.2	268.8	151.0/149.0	Neg	*y*=0.119018+34.8730×	0.998	1.17	0.96	0.05-2.5	1.3/2.0	0.9985	1.0003	0.0178	3
54	Amentoflavone	39.7	537.0	417.0	Neg	*y*=0.727280+33.3658×	0.992	1.35	1.12	0.05-2.5	2.8/5.1	0.9991	1.0044	0.0340	3
55	Chrysin	40.5	252.8	145.0/119.0	Neg	*y*=-0.0777300+18.8873×	0.999	1.46	1.21	0.05-2.5	1.5/2.8	0.9922	1.0050	0.0323	3
56	Acacetin	40.7	283.0	239.0	Neg	*y*=-0.559818+163.062×	0.997	1.67	1.28	0.02-1	1.5/2.5	0.9949	1.0011	0.0363	3

[a]R.T.: Retention time, [b]MI (*m/z):* Molecular ions of the standard analytes (m/z ratio), [c]FI (*m/z):* Fragment ions [d]r^{2}: Coefficient of determination, [e]*RSD*: Relative standard deviation, [f]*LOD*/*LOQ* (µg/L): Limit of detection/quantification, [g]*U* (%): percent relative uncertainty at 95% confidence level (k = 2), [h]IS: Internal standard, [i]Gr. No: Represents grouping of internal standards, these numbers indicate which IS stands for which phenolic compound.

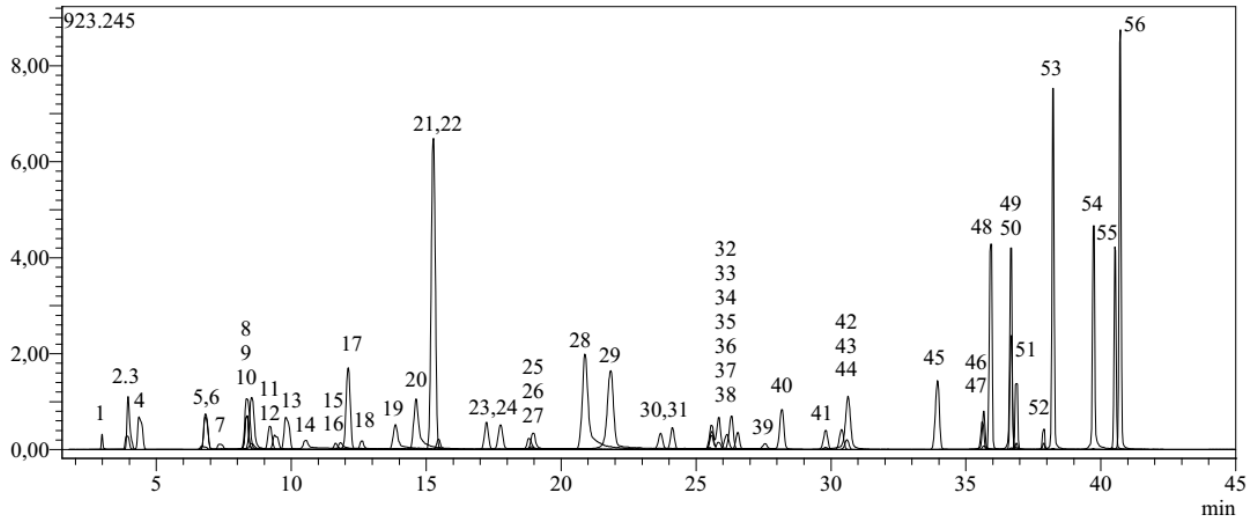

Figure 1. TIC (Total Ion Chromatogram) chromatogram of standard phenolic compounds: 1.Quinic acid 2.Fumaric aid 3.Aconitic acid 4.Gallic acid 5.Epigallocatechin 6.Protocatechuic acid 7.Catechin 8.Gentisic acid 9.Chlorogenic acid 10.Protocatechuic aldehyde 11.Tannic acid 12.Epigallocatechin gallate 13.1,5-dicaffeoylquinic acid 14.4-OH Benzoic acid 15.Epicatechin 16.VaniLlic acid 17.Caffeic acid 18.Syringic acid 19.Vanillin 20.Syringic aldehyde 21.Daidzin 22.Epicatechingallate 23.Piceid 24.*p*-Coumaric acid 25.Ferulic acid-D3-IS^{h} 26.Ferulic acid 27.Sinapic acid 28.Coumarin 29.Salicylic acid 30.Cynaroside 31.Miquelianin 32.Rutin-D3-IS^{h} 33.Rutin 34.isoquercitrin 35.Hesperidin 36.*o*-Coumaric acid 37.Genistin 38.Rosmarinic acid 39.Ellagic acid 40.Cosmosiin 41.Quercitrin 42.Astragalin 43.Nicotiflorin 44.Fisetin 45.Daidzein. 46.Quercetin-D3-IS^{h} 47.Quercetin 48.Naringenin 49.Hesperetin 50.Luteolin 51.Genistein 52.Kaempferol 53.Apigenin 54.Amentoflavone 55.Chrysin 56.Acacetin analyzed by the developed LC–MS/MS method.

Table 2: Colony color of yeast isolates on CHROMagar candida medium

Species of candida	**Colony color on Chromagar**
C. krusei	light pink colonies

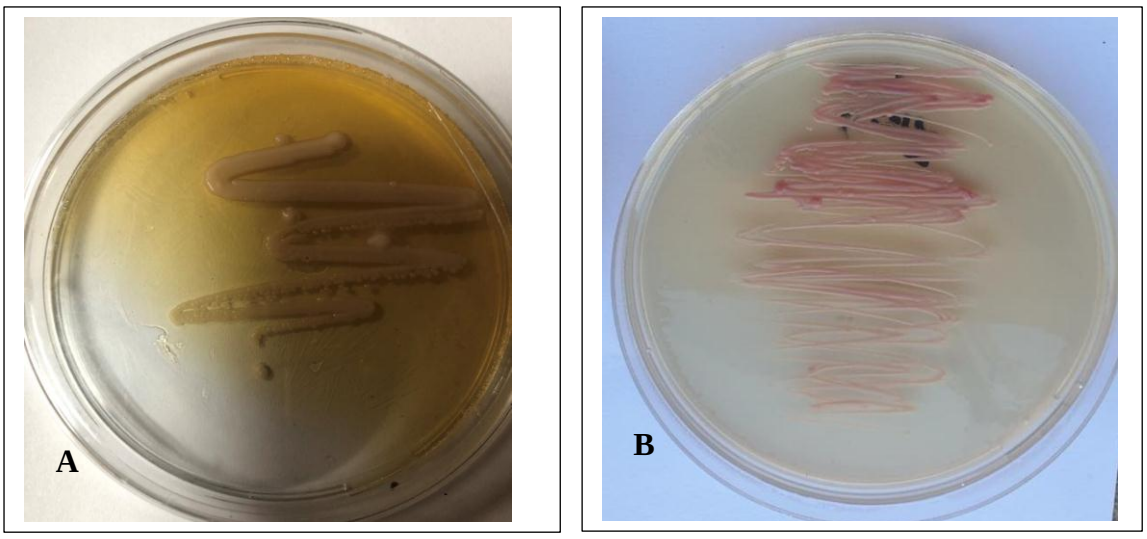

Figure2: *C. krusei* on **A.** Sabouraud Dextrose Agar (SDA) **B.** CHROMagar medium

Table 3: Antifungal susceptibility testing by Agar Well Diffusion and Disc Diffusion method

Antifungal agent				**Pomegranate Peel Extract (PPE)**			
Diameter of inhibition zone(mm)							
Disc diffusion method				**Agar Well Diffusion method**			
Econazole	**Ketoconazole**	**Miconazole**	**Nystatin**	**Aquatic**	**SDW(control)**	**Ethanol**	**DMSO(Control)**
8	**12**	**10**	**14**	**15**	**0**	**15**	**0**

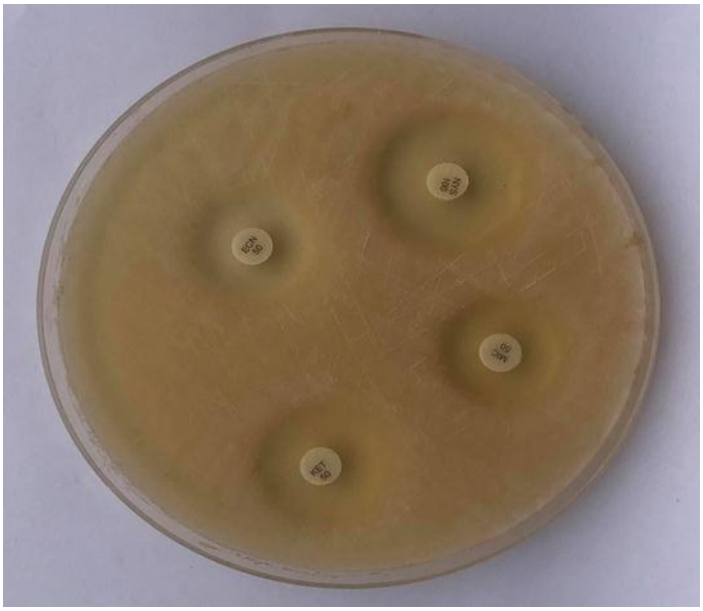

Figure 3: Susceptibility testing for antifungals by Disk diffusion method Antifungal azoles: Econazole50mg (ECN50), Ketoconazole50mg (KET50), Miconazole50mg (MIC50) and Nystatin100mg (NYS100)

Table4: Quantitative screening phytochemicals method in the ethanol and water extracts of the pomegranate by LC-MS/MS (mg analyte/g extract)

#	**Analyte**	**Pomegrenate-Alcohol**	**Pomegrenate-Water**
1	Hesperidin	0.032	0.03
2	Quinic acid	114.574	97.94
3	Fumaric aid	0.452	0.38
4	Aconitic acid	0.369	0.449
5	Gallic acid	4.204	7.117
6	Protocatechuic acid	0.029	0.049
7	Tannic acid	0.695	0.553
8	Epigallocatechin gallate	0.066	0.051
9	Cyranoside	N.D.(Not Defined)	0.014
10	isoquercitrin	0.163	0.127
11	Rutin	0.092	0.079
12	Ellagic acid	14.604	48.314
13	Cosmosiin	0.054	0.035
14	Astragalin	0.544	0.461
15	Nicotiflorin	0.324	0.301
16	Quercetin	0.012	N.D.

17	Luteolin	0.005	0.005
18	Naringenin	0.007	0.005
19	Apigenin	0.003	0.002
20	Acacetin	0.01	0.007
21	Chrysin	N.D.	0.004

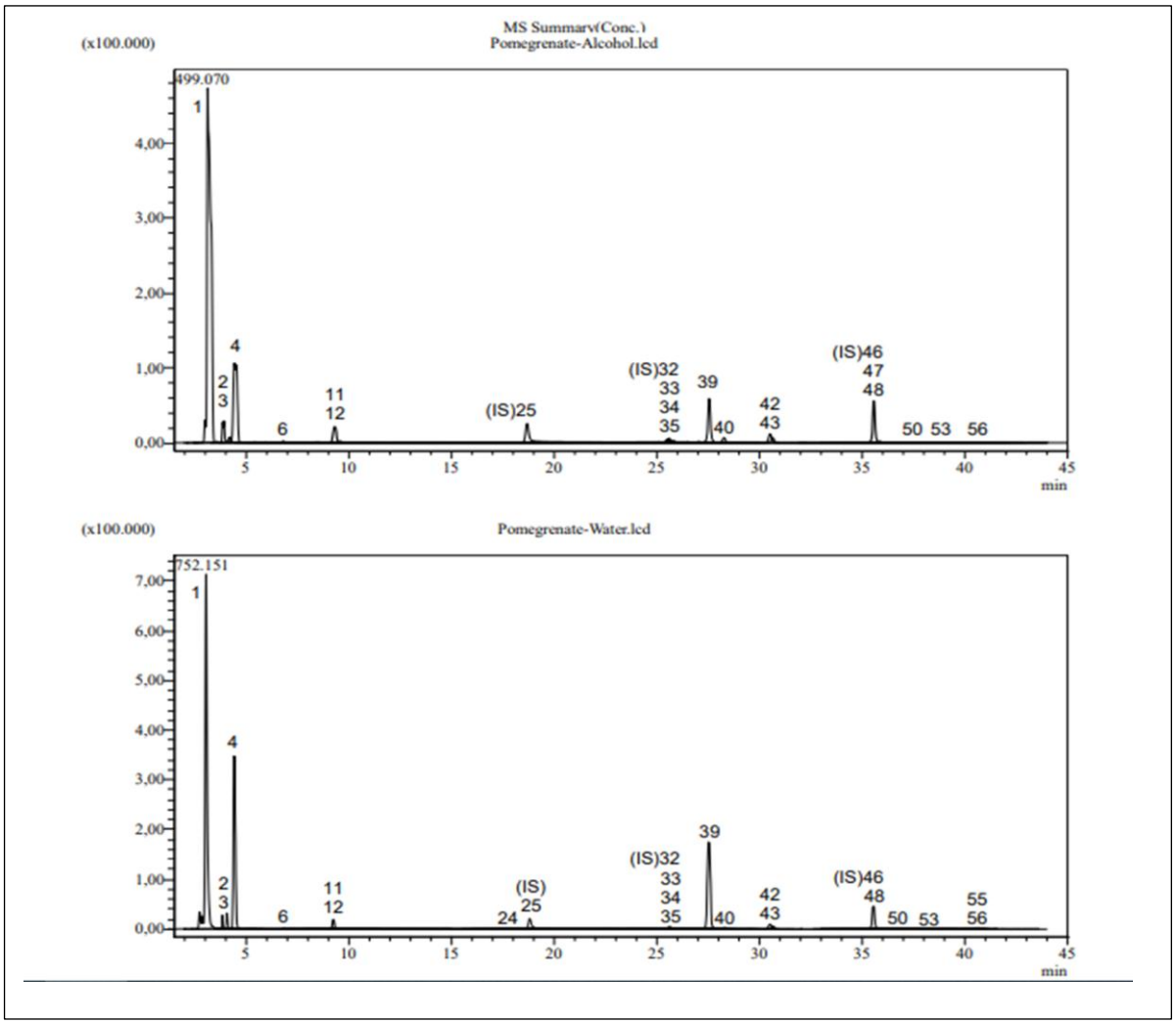

Figure4: Analytical parameters, identification, and quantification of phytochemicals of Pomegranate extracts. Alcohol and Water

References:

ABOUALIGALEHDARI, E., SADEGHIFARD, N., TAHERIKALANI, M., ZARGOUSH, Z., TAHMASEBI, Z., BADAKHSH, B., ROSTAMZAD, A., GHAFOURIAN, S. & PAKZAD, I. 2016. Anti-biofilm Properties of Peganum harmala against Candida albicans. *Osong Public Health Res Perspect*, 7**,** 116-8.

AIBINU, I., ADENIPEKUN, T., ADELOWOTAN, T., OGUNSANYA, T. & ODUGBEMI, T. 2006. Evaluation of the antimicrobial properties of different parts of Citrus aurantifolia (lime fruit) as used locally. *Afr J Tradit Complement Altern Med*, 4**,** 185-90.

ANIBAL, P. C., ALVES PEIXOTO, I. T., FOGLIO, M. A. & HÖFLING, J. F. 2013. Antifungal activity of the ethanolic extracts of Punica granatum L. and evaluation of the morphological and structural modifications of its compounds upon the cells of Candida spp. *Brazilian Journal of Microbiology* 44**,** 839-848.

BAKKIYARAJ, D., NANDHINI, J. R., MALATHY, B. & PANDIAN, S. K. 2013. The anti-biofilm potential of pomegranate (Punica granatum L.) extract against human bacterial and fungal pathogens. *Biofouling*, 29**,** 929-37.

BASSIRI-JAHROMI, S., KATIRAEE, F., HAJIMAHMOODI, M., MOSTAFAVI, E., TALEBI, M. & POURSHAFIE, M. R. 2015. In Vitro Antifungal Activity of Various Persian Cultivars of Punica granatum L. Extracts Against Candida species. *Jundishapur Journal of Natural Pharmaceutical Products*, 10.

BASSIRI-JAHROMI, S., POURSHAFIE, M. R., ARDAKANI, E. M., EHSANI, A. H., DOOSTKAM, A., KATIRAE, F. & MOSTAFAVI, E. 2018. In Vivo Comparative Evaluation of the

Pomegranate (Punica granatum) Peel Extract as an Alternative Agent to Nystatin against Oral Candidiasis. *IJMS* 43.

CAI, L.-Y., SHI, F.-X. & GAO, X. 2011. Preliminary phytochemical analysis of Acanthopanan trifoliatus (L.) Merr. *Journal of Medicinal Plants Research,* 5**,** 4059-4064.

CHROMART 2020. CHROMOGENIC CANDIDA AGAR. *Biolife Italiana S.r.l., TS-548005 rev 1* 1-3.

DAHHAM, S. S., ALI, M. N., TABASSUM, H. & KHAN, M. 2010. Studies on Antibacterial and Antifungal Activity of Pomegranate (Punica granatum L.) *American-Eurasian J. Agric. & Environ. Sci.,* 9**,** 273-281.

EL-DESOUKY, T. A. 2021. Evaluation of effectiveness aqueous extract for some leaves of wild edible plants in Egypt as anti-fungal and anti-toxigenic. *Heliyon,* 7**,** e06209.

ENDO, E. H., UEDA-NAKAMURA, T., NAKAMURA, C. V. & FILHO, B. P. 2012. Activity of spray-dried microparticles containing pomegranate peel extract against Candida albicans. *Molecules,* 17**,** 10094-107.

FOSS, S. R., NAKAMURA, C. V., UEDA-NAKAMURA, T., CORTEZ, D. A., ENDO, E. H. & DIAS FILHO, B. P. 2014. Antifungal activity of pomegranate peel extract and isolated compound punicalagin against dermatophytes. *Foss et al. Annals of Clinical Microbiology and Antimicrobials* 13**,** 1-6.

FOTOS, P. G., VINCENT, S. D. & HELLSTEIN, J. W. 1992. Oral candidosis. Clinical, historical, and therapeutic features of 100 cases. *Oral Surg Oral Med Oral Pathol,* 74**,** 41-9.

GANDHI, T. N., PATEL, M. G. & JAIN, M. R. 2015. AntifungAl SuSceptibility of cAndidA AgAinSt Six AntifungAl drugS by diSk diffuSion Method iSolAted froM VulVoVAginAl cAndidiASiS. *Int J Cur Res Rev* | Vol 7 20-25.

HABIB, K. A., NAJEE, E. N. & ABOOD, M. S. 2016. Identification of Candida species Isolated From Vulvovaginal Candidiasis Patients by Chromgen agar and PCR-RFLP Method. *Baghdad Science Journal,* 13**,** 291-297.

LEITE, S. J., DE OLIVEIRA, F. M., ALMEIDA, L. M. S., GUSMÃO, M. A., CHEDIER, L. M., VASCONCELOS, E. G., SILVÉRIO, M. S. & PINTO, P. D. 2014. In vitro assessment of the antimicrobial effects of pomegranate (Punica granatum L.) peel decoction on saliva samples. *Journal of Basic and Applied Pharmaceutical Sciences,* 35**,** 25-28.

LIGUORI, G., GALLÉ, F., LUCARIELLO, A., ONOFRIO, V. D., ALBANO, L., MAZZARELLA, G., D'AMORA, M. & ROSSANO4, F. 2010. Comparison between multiplex PCR and phenotypic systems for Candida spp. identification. *NEW MICROBIOLOGICA,* 33**,** 63-67.

MOHAMED, B. J. & THWANI, A. N. A. 2010. The susceptibility test of vaginal yeasts and their relationship with the age in Iraqi women. *Baghdad Science Journal,* 7**,** 1174-1180.

MOHSIN, Z. A. & ALI, W. A. S. 2021. Antagonistic Activity of Bacteriocin-producing Lactobacillus Against Candida spp. *Iraqi Journal of Science***,** 2153-2162.

NADEEM, S. G., HAKIM, S. T. & KAZMI, S. U. 2010. Use of CHROMagar Candida for the presumptive identification of Candida species directly from clinical specimens in resource-limited settings. *Libyan J Med,* 5.

OTHMAN, K. I., ABDULLAH, S. M., ALI, B. & MAJID, M. 2018a. Isolation and Identification Candida spp from Urine and Antifungal Susceptibility Test. *Iraqi Journal of Science,* 59.

OTHMAN, K. I., ABDULLAH, S. M., ALI, B. & MAJID, M. 2018b. Isolation and Identification Candida spp from Urine and Antifungal Susceptibility Test. *Iraqi Journal of Science,* 59**,** 1981-1988.

OZCAN, K., ILKIT, M., ATES, A., TURAC-BICER, A. & DEMIRHINDI, H. 2010. Performance of Chromogenic Candida agar and CHROMagar Candida in recovery and presumptive identification of monofungal and polyfungal vaginal isolates. *Med Mycol,* 48**,** 29-34.

QIN, F., WANG, Q., ZHANG, C., FANG, C., ZHANG, L., CHEN, H., ZHANG, M. & CHENG, F. 2018. Efficacy of antifungal drugs in the treatment of vulvovaginal candidiasis: a Bayesian network meta-analysis. *Infect Drug Resist,* 11**,** 1893-1901.

RÍOS, J. L., RECIO, M. C. & VILLAR, A. 1987. Antimicrobial activity of selected plants employed in the Spanish Mediterranean area. *J Ethnopharmacol,* 21**,** 139-52.

RODRIGUEZ-LEGUIZAMON, G., FIORI, A., LOPEZ, L. F., GOMEZ, B. L., PARRA-GIRALDO, C. M., GOMEZ-LOPEZ, A., SUAREZ, C. F., CEBALLOS, A., VAN DIJCK, P. & PATARROYO, M. A. 2015. Characterising atypical Candida albicans clinical isolates from six third-level hospitals in Bogota, Colombia. *BMC Microbiol,* 15**,** 199.

ROSCETTO, E., CONTURSI, P., VOLLARO, A., FUSCO, S., NOTOMISTA, E. & CATANIA, M. R. 2018. Antifungal and anti-biofilm activity of the first cryptic antimicrobial peptide from an archaeal protein against Candida spp. clinical isolates. *Sci Rep,* 8**,** 17570.

SHAOKAT, S. S., HAMEED, H. A. & MOHAMMAD, H. J. 2007. Anti-fungal Activity of Punica Granatum I.peels Powder and Extracts from Pathogenic Samples. *Iraqi J.Pharm.Sci.* , Vol.16 12-20.

SRINIVASAN, D., NATHAN, S., SURESH, T. & LAKSHMANA PERUMALSAMY, P. 2001. Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. *J Ethnopharmacol,* 74**,** 217-20.

YILMAZ, M. A. 2020. Simultaneous quantitative screening of 53 phytochemicals in 33 species of medicinal and aromatic plants: A detailed, robust and comprehensive LC–MS/MS method validation. *Industrial Crops and Products,* 149**,** 112347.

YILMAZ, M. A., ERTAS, A., YENER, I., AKDENIZ, M., CAKIR, O., ALTUN, M., DEMIRTAS, I., BOGA, M. & TEMEL, H. 2018. A comprehensive LC-MS/MS method validation for the quantitative investigation of 37 fingerprint phytochemicals in Achillea species: A detailed examination of A.

coarctata and A. monocephala. *J Pharm Biomed Anal,* 154**,** 413-424.

YONIS, R. W., LUTI, K. J. K. & AZIZ, G. M. 2019. STATISTICAL OPTIMIZATION OF CHITIN BIOCONVERSION TO PRODUCE AN EFFECTIVE CHITOSAN IN SOLID STATE FERMENTATION BY Asperigellus flavus. *Iraqi Journal of Agricultural Sciences,* 50**,** 916-927.