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# Molecular typing ofdandruff pathogens and evaluated the antifungal activity of plant extracts

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**Abstract : Background** : Dandruff is a common dermatological problem, dispersed flaking of the scalp and hair. Various intrinsic and environmental factors such as skin surface fungal colonization, individual susceptibility, are affecting.
The etiology of dandruff is not well understood. The study aims to verify the unequivocal understanding of the fungal relationship with dandruff by identification of filamentous fungi, *Candida* and survey of the bacterial companioning dandruff of hair samples and investigate the antifungal activity of water extracts of some medicinal plants in isolated fungi. A total of 280 hairs scalps samples,and hair swabs were collected from patients attenuated in Hilla hospitals and private clinics inthe Babylon province (n= 152 hair samples,n= 74 dandruff samples,n= 54 scalp swabs).Clinical samples were cultured on Sabouraud dextrose agar (SDA) medium with / without antibiotics based on stander cultured methods.*Candida* spp. was preliminary identified based on CHROMagar medium. Molecular typing of isolated yeasts via amplification ITS region and sequence analysis and multiple alignment was performed and constricted the phylogeny tree.
**Keyword:**Dandruff, Molecular typing, fungi,Plant extract activity.

## Introduction

Scalp skin has unique predisposing factors like hair follicles density ,moist ,dark and warm environment ,these factors make it susceptible to superficial mycotic infections such as dandruff,dermatitis and tinea captis[1].Dandruff is extremely common, affecting close to 50% of the world's population[2].It's characterize white or gray flakes, occurred in patches or scattered on the scalp,usually accompanied by itching[1,3,4].The health care orientation, high costs.In USA the cost exceeded $1.4 billion in the United States in 2004 and causes patient's psychological suffering[5]. Many factors were suspected with occurring of dandruff,some of them are intrinsic and environmental factors, such as skin surface fungal colonization, individual susceptibility, genetic, patient immunity and interactions between these factors, led to the dandruff occurrence[6]. The yeasts *Malassezia* and *Candida* may aggravate atopic dermatitis due to an allergic reaction and led to damaging the surface of the hair, scalp may share in the development of dandruff[7].

The etiology of dandruff appears to be dependent upon many factors: scalp gland secretions, head lice, microfloral metabolism, individual susceptibility and their interactions[8-10], but the exact underlying cause of dandruff is unknown[11]. Fungi were one of the most suspected causative agents of scalp health problematic worldwide[12].

Unfortunately, most of previous studies consider the dandruff were given more attention to the *Malassezia* spp is the unique causes.A secreted lipase of *Malassezia* is an associated virulence factor of

development of dandruff, and quite neglected others fungi[13-16].Faergemann[17] pointed to there is little information about the distribution and colonization of *Candida*spp. of skin in patients with atopic dermatitis. The Deutromycete,Ascomycetes and Basidiomycete yeast are colonizing the human scalp and hairs and get their role in the development of dermatitis as primary or secondary pathogens[18].

The study aims to verify the unequivocal understanding of the fungiassociated with dandruff by identification of filamentous fungi, yeastsand bacteria companioning dandruff of hair samples, and investigate the antifungal activity of aqueous extracts of some medicinal plants in isolated fungi.

## 2.Material and Methods

Two hundred and eighty dandruff samples (152 hair samples and 54 swabs and 74 scraps) were collected from patients at different ages and gender, those were previously diagnosed by a physician for the presence of hair dandruff symptoms.

Clinical specimens (piece of hairs, flakes and swabs were cultured into two sets:first one was cultured on Sabouraud's dextrose agar medium(SDA)without cyclohexamide and incubated for 24-48h 30˚C. Second set with cyclohexamide and incubated at 28-30˚C for 1-3 weeks.After incubation periods, single yeast and fungal colonies were isolated in pure cultures,phenotypic identification was performed based on standard methods[19,20]. The subcultures in each isolate were preserved in slant SDA media for future tests[21].While the bacterial isolation , purification and identification on the blood agar base and manitol salt agar based on McFadden[22].

### 2.1.CHROMagar test

This test was used for preliminary identification most of Deutromycetes, Ascomycetes and Basidiomycetes yeast. Each single colony yeast with white –cream color were pickup and streaking on CHROMagar medium[23].All the plates were culturedand incubated at 30˚C for 24-48h.While the red colonies were directly identified as *Rhodotorulla.*

### 2.2.**Lipase andPhospholipase production assay**

*Candida*specieswere screened for the production of extracellular lipase and phospholipase activity by growing them on asubstrate with SDA: tween 80 medium and egg yolkrespectively and incubated at 37˚C for 48 h.after which the colony diameter plus precipitation zone was measured for each isolate. Calculation of the zone of phospholipase activity was performed according to Price *etal*.[24].

### 2.3.Plants crud extraction and preparation

Plant material included of *Lawsonia inermis*( leaves of Henna), *Eugenia caryophyllus* (floral buds of Clove),*Cinnamomum verum* (bark) ,*Camellia sinenesis* (leaves) and *Eucalpytus globules*(leaves) were collected from markets.They were identified by the taxonomist in the biology department ,All women college for Science in Babylon University .The extracts were prepared based on Vijayakumar*et al.*[25].Theserial concentration (1%,4%,8%)were evaluated their antifungal activities against dermatophytes and yeasts.

### 2.4.Evaluate the antifungal activities

Brief description of the method,pour about 20 mlSDA to Petri dish,0.2ml of cell suspension($1x10^6$) was spread on the surface of SDA and left it to been absorption in the medium.Wells (0.5cm diameter) in SDA were performed by cork pore and filled each well of SDA with 100 µl of plant extract, incubate 28-30˚C for 24-48 h, the inhibition zone was measured by metric ruler.All tests were performed in triplicate[26].

### 2.5.PCR and sequencing analysis:

The phenotypic identification of fungi under interestedwasconfirmedby simple PCR by universal primer pair ITS5/ITS4.OneµLofDNA(20µg/ml)fromeach of 24 isolates were mixed with a PCR mixture (final reaction volume 25 µL) consisted of 12 µL of 20x Master Mix (Promega),2 µL of primers (10 pmole) and restmolecular-gradewater. The PCR conditions and gel electrophoresiswere performed based on Imran and Al-Asadi[27].

Representative 22PCR products offungi were subjected to sequencing analysis in the Macro gene Lab. USA. Pairwisealignmentsequenceswere compared with theBLASTdatabase. The phylogenetic tree (UPGM) based on sequencing were constructed employing the Mega 6 software, multiple alignment sequencesbased on BioEdit software was performed[28].

### 2.4. Statistical analysis

All statistical analysis was undertaken using factorial experimental randomized block design. A *P*.value < 0.05 was considered significant.

## 3.Results:

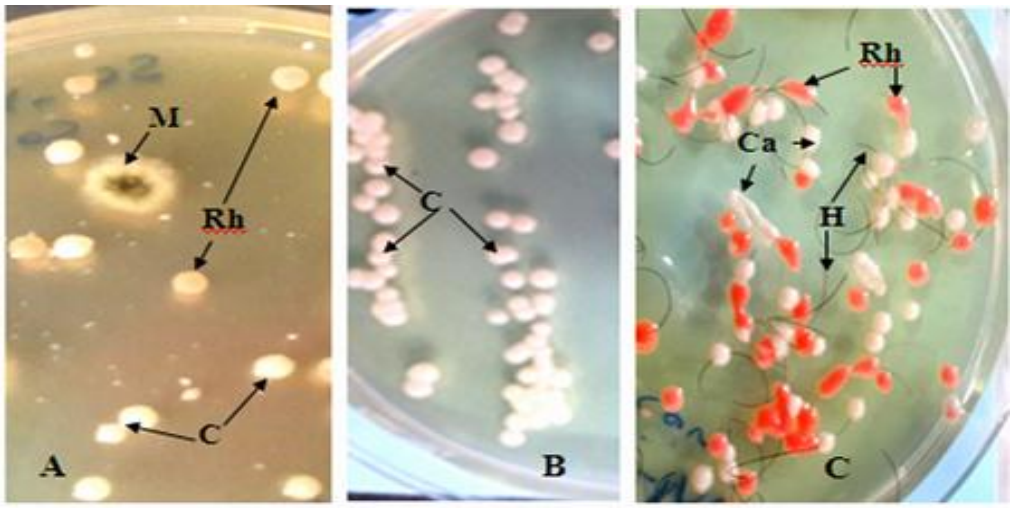
### 3.1.Fungal survey :

A total of 2313 colonies (1591 yeast colonies,722 filamentous fungi colonies)was isolated from clinical specimens (hair, flakes, scalp swabs).The percentage of filamentous fungi were summarized in table (1), the results showed that the *Aspergillus flavus* and *Asp.fumegatus* were the highest molds while the *T. rubrum* and *M.canis*were the highest dermatophytes fungi.The survey of bacteria with dandruff cases showed a frequency percentage of *S. aureus* and *S. epidermidis* 35.1 % for each.

**Table (1): Summarized the appearance and frequency percentage of filamentous fungi isolated from clinical samples**.

| Fungi | No. of samples | No. of colonies | Percentage of appearance | Percentage of frequency |
|---|---|---|---|---|
| Alternaria spp | 28 | 42 | 10 % | 5.81 % |
| Aspergillus flavus | 82 | 193 | 29.28 % | 26.73 % |
| Asp. Fumigates | 33 | 169 | 11.78 % | 23.40 % |
| Asp. Niger | 45 | 106 | 16.07 % | 14.68 % |
| Asp. Terreus | 1 | 1 | 0.35 % | 0.13 % |
| Asp. Parasiticus | 5 | 17 | 1.78 % | 2.35 % |
| Macrosporum | 6 | 21 | 2.14 % | 2.90 % |
| Penicillium spp | 18 | 42 | 6.42 % | 5.81 % |
| Rhizopusstolonifer | 1 | 1 | 0.35 % | 0.13 % |
| T. interdigetale | 1 | 3 | 0.35 % | 0.41 % |
| T. rubrum | 5 | 125 | 1.78 % | 17.31 % |
| T. verrucosum | 1 | 2 | 0.35 % | 0.27 % |
| Total | 226 | 722 | 80.65 % | 99.93 % |

Data on the growth of *Candida*spp and*Rhodotorulla* on hairs planted on SDA comparison with direct scalp's swab streaking and scattered flakes on SDA medium are presented in figure (1).Both yeast grew well in whole hairs.*Candida* spp and *Rhodotorulla* demonstrated more rapid and abundant growth of hairs than SDA medium after 24-48h,most frequent and vital colonies on hairs,may supported growth both *Candida* and *Rhodotorulla* compare with their growth on SDA (Figure 1) .




**Figure(1):Abundant of yeasts grown on clinical samples :A=scatter of flakes, B= Swab streak from dandruff cases, C= Hairs infected by dandruff.(Rh= *Rhodotorulla*, C=*Candida*,M=Mould,H=Hairs).**

The percentage of appearance *Candida* spp. were summarized in table (2) ,the results showed *C.parapsilosis* was the highest yeast (28.75% (80/280), while the frequency of percentage of it was 25.14% (400/280). The appearance of *R.mucitaginosa* was 7.14% (20/280) and the frequency percentage was 8.79% (140/280).

**Table (2): Summarized the Percentage of appearance and frequency values of yeasts and their colors on CHROMagar .**

| ***Candida* spp** | CHROMagar | **Samples No.** | **Colonies No.** | **Percentage** | **Frequency** |
|---|---|---|---|---|---|
| *C. albicans* | Light green | 17 | 125 | 6.07 % | 7.9 % |
| *C. glabrata* | Pale pink | 20 | 250 | 7.14 % | 15.71 % |
| *C. intermedia* | Dark purple | 36 | 300 | 12.85 % | 18.85 % |
| *C. kruzei* | Pink | 50 | 350 | 17.85 % | 21.99 % |
| *C.parapsilosis* | Cream – pale | 80 | 400 | 28.57 % | 25.14 % |
| *C. tropicalis* | Metallic blue | 10 | 26 | 3.57% | 1.63 % |
| *R.mucitaginos* | Still Red | 20 | 140 | 7.14 % | 8.79 % |
| Total | | 233 | 1591 | 83.19 % | 99.96 % |

### 3.2.Lipase and phospholipase production tests

Our results showed that *C.albicans*, *C.tropicalis* and *C.parapsilosis* gave positive results for their ability to produce lipase and Phospholipase invitro. The singer of lipase and Phospholipase production is a precipitation around the colony after an incubation period of 72 h at 30˚C (Figure 2),otheryeast showedpositive tests (data not showon).

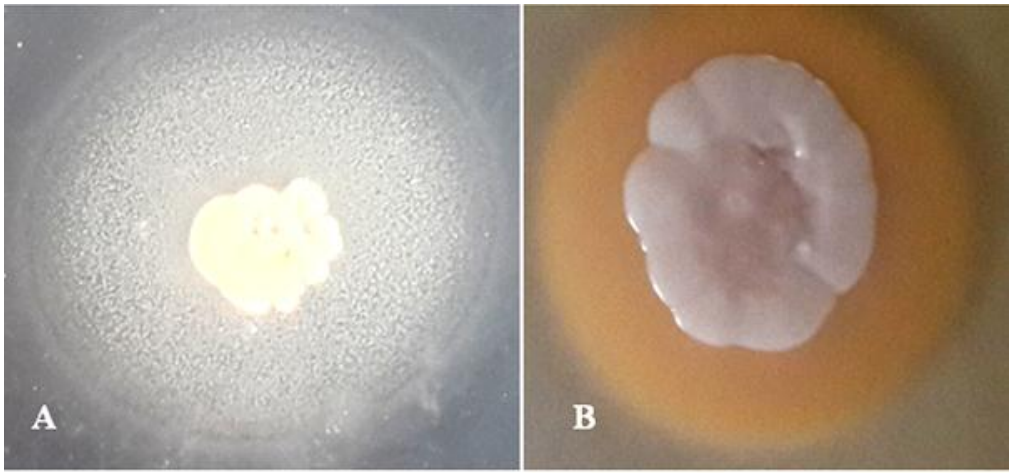



**Figure (2) : Lipase(A) and Phospholipase(B) production singe as precipitation around *Candida* colony at incubation for 72h,30C.**

### 3.3.Crude of plant extract activity:

The activity of plant extract crude showed significant inhibition in the colony diameter of fungi.Figure(3) showed the variation of inhibition zones based on plant species: the aqueous extract of cloves was more effective (0.62 mm),followed by *Cinnomumm* ,*Eucalyptus*, green tea ,henna (0.25,0.28,0.29,0.23) respectively.

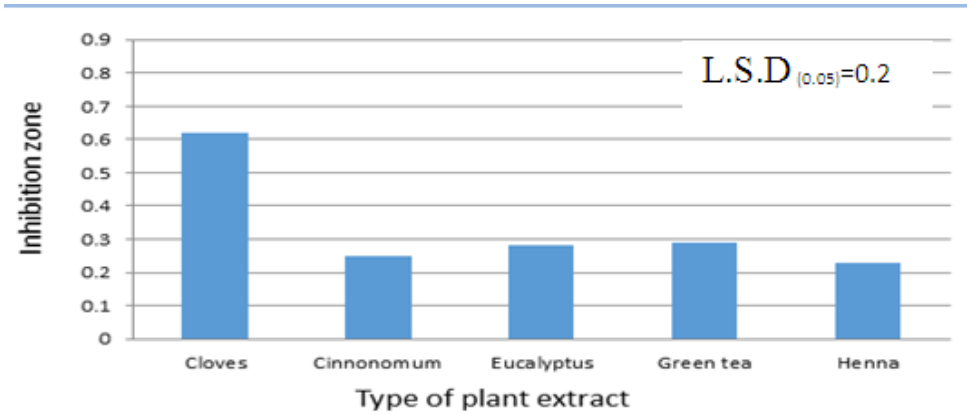



**Figure (3) : Effect of the extract type in the inhibition zone of dermatophytes and yeast .**

The sensitivity of fungi to the effects of plant extracts were showed significant difference :*T.rubrum* more sensitive followed by *M.canis, C.albicans, C.glabrata, C.parapsilosis, C.intermedia* and*R.mucitaginosa*respectively ,(Figure 2).also the concentration 8% showed more effective than 2% and 4% respectively (data not showon).

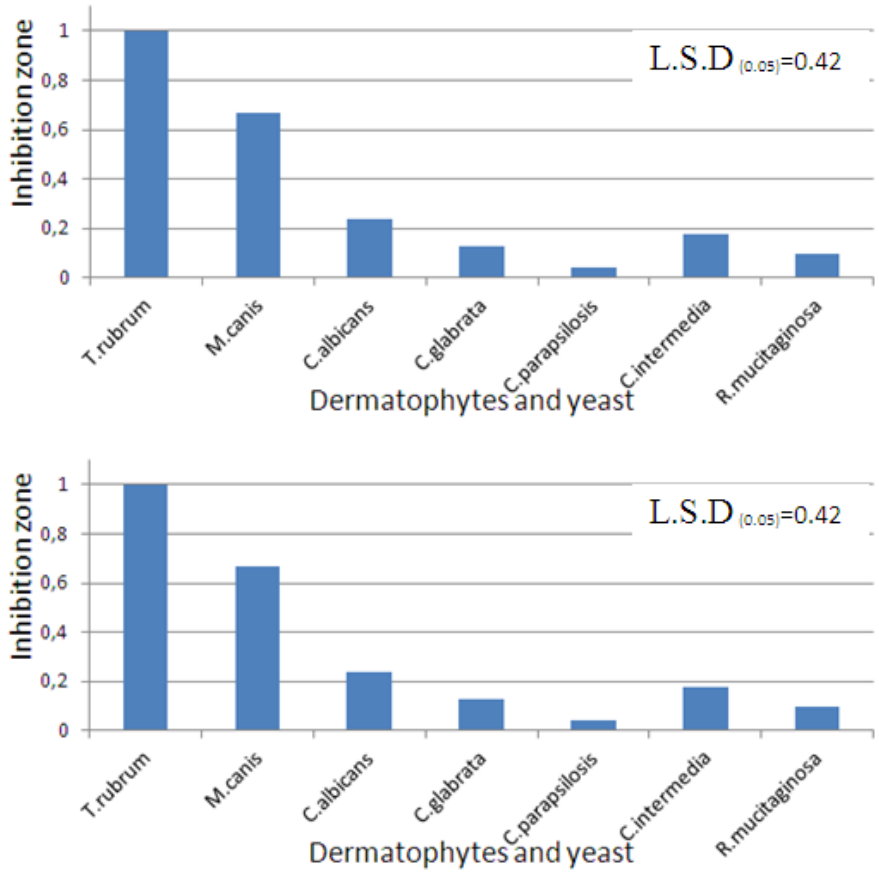



**Figure (**2 **) : Fungal sensitivity to the crude of plant extract determined based on the inhibition zone of dermatophytes and yeast growth.**

Antfactivitungalof five extracts were comparable with three reference antifungals (Econazole (ECN), Miconazole (MCL) and 5-fluorocytosine AFY),the extract of clove gave antifungal activity higher than that of references (ECN, MCL and AFY),while the other four plant extract gave lower antifungal activities than that reference antifungals (Figure 3).

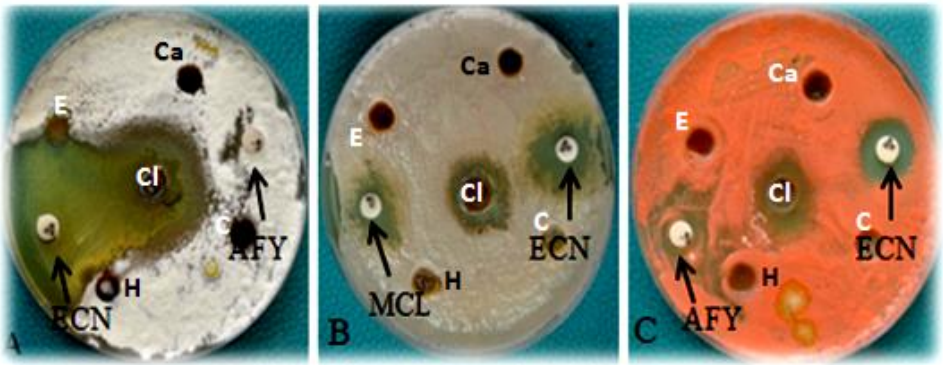



**Figure (3) : Antifungal activities of five plants extracts: Cl= Cloves ,E=*Euclaptus*, C= *Cinnamomum*, Ca= *Camellia.* H=hana, ECN, MCL, AFY=reference antifungals, A=culture *T.rubrum*, B= culture of *C.intermedia* and C=culture of *R.mucitaginosa***

### 3.4.PCR and sequencing assay

The results of amplification ITS1-5.8S –ITS2 and flanking of primer pairs ITS5/ITS4 showed variation in the amplicons sizes of 24 isolates dermatophytes and yeasts for each(Figure4,A&B). *T.mentogrophytes* isolates (680,650 bp). *T.rubrum* isolates (780,800bp).

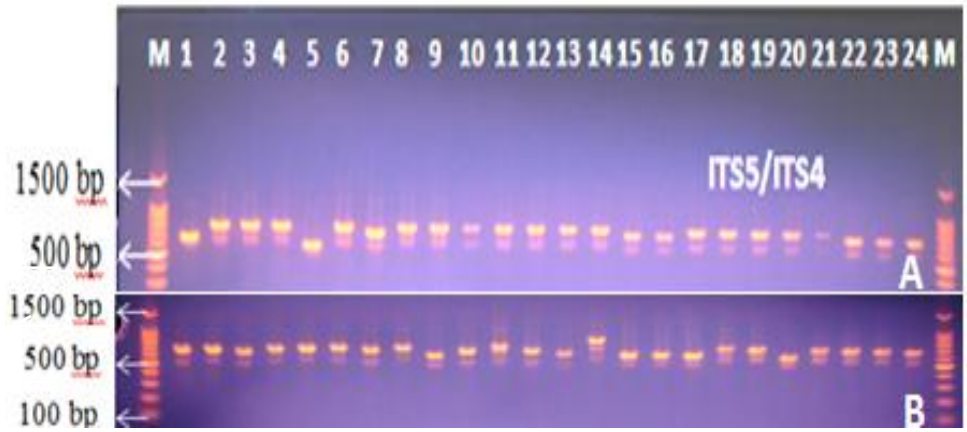



**Figure (4 ) : Agarose gel electrophoresis of PCR products: A. Fordermatophytesspecies isolateamplified by pair primer ITS5/ITS4: Lane M= Molecular marker100 bp.; lane one *T.mentogrophytes* isolates (680 bp.). Lanes 2-4,6,8-14,17-21 *T.rubrum* isolates (800bp), Lanes 5 *T.mentogrophytes* isolates (650bp).Lanes 7,15,16,22-24 *T.rubrum* isolates (780bp).B. For*Candida* species isolate. Lane M = Molecular marker 100 bp.; Lanes 1-2,4-8,11,18-19,21-24:680 bp.(*C.tropicalis*); Lanes 3,9,13,15-17,20 630 bp.(*C.parapsilosis* ); Lanes 14: 720 bp.(*C.kruzea*); Lanes 10,12 : 550 bp.(*C.albicans*)**

3.5.**Multiple alignment of 17 sequences of ITS** region

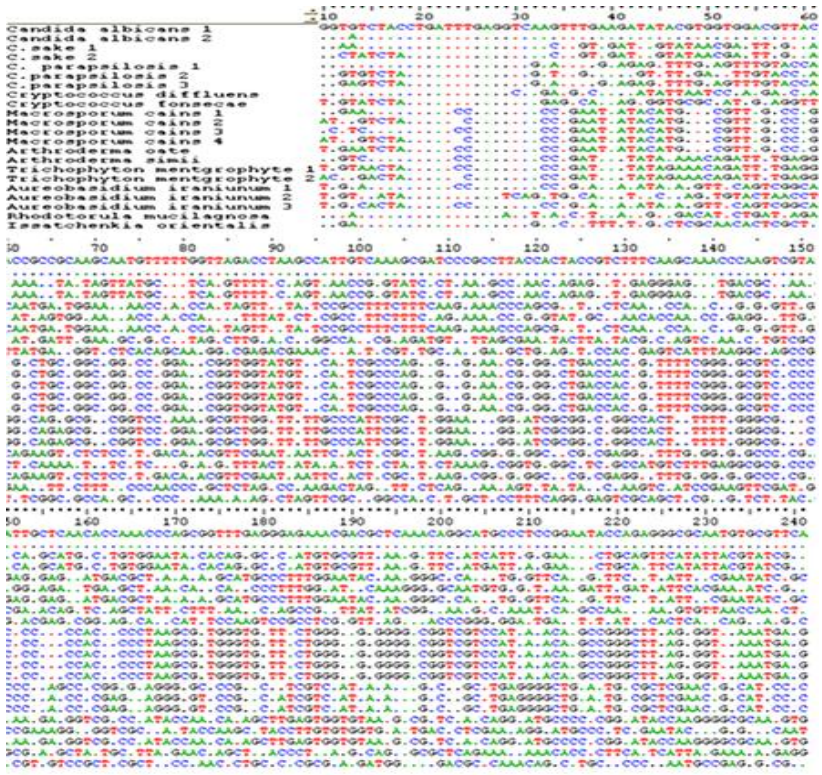


**Figure (5): The Multiple-alignment of Sequence analysisof ITS region amplified by ITS5/ITS4 for suspected fungal dandruff pathogens.**

The results of themultiple alignment analysis based on BioEdit software performed for 22 isolates of yeast:*C.albicans* and *Cryptococcus*spp ,*C.parapsilosis Aureobasidium iranianum*,*Issatchenkia orientalis*, *R.mucitaginosa* and dermatophyte (Figure5). Each set of isolates of*Candida* sppwas showed high similarity to leading sequences with some mutation or substitutions, these sequence variations were indicated to microevolution in each set of isolates.

### 3.6. Phylogeny tree:

The Phylogenetic tree(UPGM) for 21 fungal species was constructed based on sequences of ITS region. Thefungal specieswere isolated from dandruff samples showed closed related intra-isolates groups as in Figure (6), many clusters of closely related fungal species The dermatophytes occurred in neighbored clusters,the same relationship between *C.albicans*,*C.sake*and*C.parapsilosis* were close to either.The Basidiomycetes yeasts separated their clusters of previous clusters depended on their sequences.

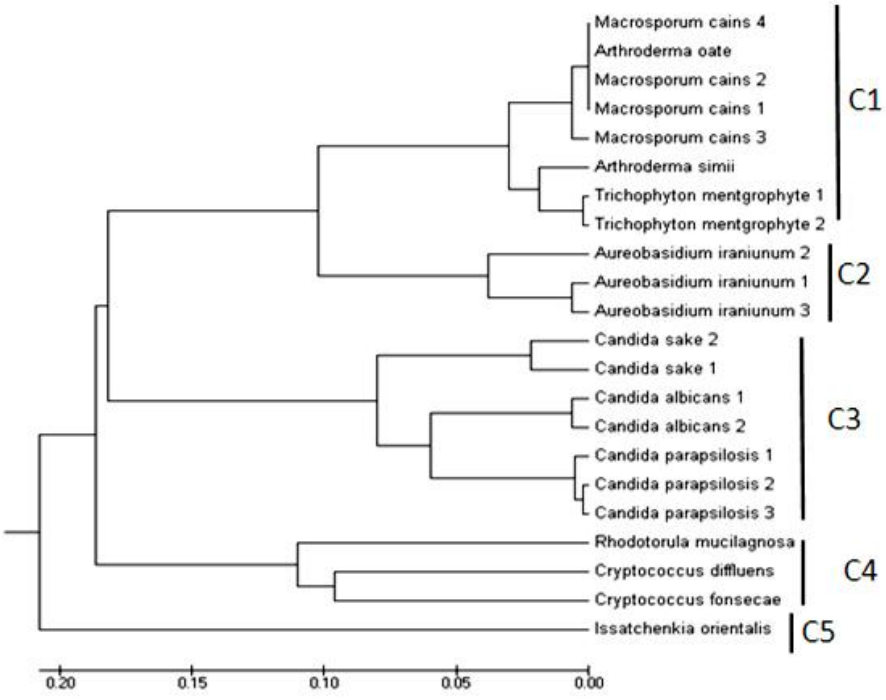



**Figure (6): Phylogeny tree (UPGM) based on sequence of 21 fungalspecies suspected as dandruff pathogens occurred in five clusters (C1=dermatophyte,C2 and C4 =Basidiomycetes yeast,C2=Deutromycets yeast, C5= Ascomycetes yeast).**

## 4.Discussions:

Mycotic pathogens were one of the suspected on the scalp of dandruff patients, most of previous works considered *Malassezia* sppas the most common pathogen, others works reportedto role of combined factors, but inthe general mechanism of occurrence of the disease is not clearly understood[9,10,15,16]

This studyidentified 12 fungal species issociated with all clinical samples :(4dermatophytes, 3*Candida*, one *Aureobasidium iranianum*,*Issatchenkia orientalis* and *R.mucitaginosa* for each, 2*Cryptococcus*)based on molecular assays (Figures 4-6). Most of these dermatophytic fungi cause dermatitis,our results agree with the results of studies ofTan,[29]; Jain *etal*., (30);Abastabar*et al*.[31].

Our study was aimed to evaluate antifungal activities of five plant extracts invitro against suspected dandruff pathogens , Clove extract showed signeficantlyantifungal activity more than others extracts,either than reference antifungals drugs such as ECN, MCL and AFY (figure 3).the microbial inhibition activity of Clove depende on it's composion of flavenoides and cartenoides ,this finding agreed with[32],our results recommended

to evaluate the clinical efficacy and safety of Clove extract as anti-Dandruff Shampoo based on their size of inhibition zone compare with reference drugs .

This study was verified that most of yeast:*C.albicans ,C.parapsilosis,C.tropicales* and *R.mucitaginosa* had the ability to produced lipase and Phospholipase, this result was agree with recent studies were reported that lipasecausing dermal inflammation and tissue damageand play a key role in the lifestyle of opportunistic yeast[28, 33-37].The high frequent of dermatophyte fungi and yeastsuch as*Microsporum*, *Trichophyton* and *Candida*in this study may take their role in the attacked of scalp hair follicle and led to development dandruff, thisexplanation agreed with Herbert[38] andZinkevicien*et al.*[39],also*Staph.aureus* and *Staph.epidermidis*,were considered an opportunistic pathogen has the ability to colonize in different niches[40].

Our finding results, in particularbased on the abundant growth of *Candida* and*Rhodotorulla* on hairs, scalp and flakes and the ability of these yeasts for lipase and Phospholipase production(Figure 1, 2).This result tends to confirm the virulence of *Candida* spp and *Rhodotorulla* are considered to be the most virulent compared with *Malassezia* spp. has been questioned by[13-17],who studied only *Malassezia* havevirulence factor depended on lipase production.

Our finding highly frequent and abundant growth of *Candida* spp,,*R.mucitaginosa* and *Cryptococcus*with absent of *Malassezia* spp.in all clinical samples under interest (Figure 1).This finding was supportedby the results of Golubev[37]and Zinkevicien*et al.*[39],the found negative relationship between *Candida* spp and *Malassezia* spp.when they grew in the same niche,Both*Malassezia* and *Candida* were not found together in any of the samplesdue to different growth rate, *Candida* had an antagonist role against *Malassezia* spp. and*Candida* spp. have the ability to produce kill factors(mycocin as lethal substance) led to inhibition and killed *Malassezia* spp.The high growth rate of *Candida* more than *Malassezia* spp this property led to overgrowth of *Candida* against *Malassezia* spp.which has no special requirement for growthmedia[37,39,40].These justifications were explained the predominance of *Candida* spp,*R.mucitaginosa* and *Cryptococcus* spp. wedetermined in this study.

Our conclusion, based on this results and review,We think it was impossible to note that were considered both *Candida* and*Rhodotorulla* as harmless yeast with dandruff patients from all previous works. Andwe consider the dermatophytes,*Candida* and *Rhodotorulla* were important dandruff mycotic pathogens,and the current data refute their contention about the consideration the *Malassezia* spp as the main dandruff pathogen.More studies are required to conform our results about the pathogenic role of fungi act as exacerbating factors in dandruff.

**Ethical approval**

Author hereby declared that all the actions have been examined in the studies were approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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