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Effects of some plants Extracts on Growth of some Candida Species Isolated from Oral Thrush, Thalassemia, Orthodontics Patients

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Abstract

Due to the increase of microbial resistant to antibiotics in addition to recurrent fungal infections, the attentions in medicinal plants and herbs are increased. 52 samples of oral swabs were gathered from patients (male and female) with age average equal to 25.5 with different levels of clinical improvement (thrashing= 18, thalassemia = 15, orthodontics= 19). The samples were obtained from Diyala General Hospital and some health centers in Diyala province\Iraq. *Candida albicans* was the most predominant spp. among different specimens' groups fallowed by *C. trupicalis, C. glabrata* and lastly *C. krusei. Candida* spp are most prevalence in thalassemia patients fallowed by premature babies and orthodontics oral swabs, *C. albicans* were the most affected by Clutrimazole, *C. trupicalis* and *krusei* were resistant to Fluconazole *C. krusei* was the least affected by antifungals were used in this study. The culture results on media contain plant extracts showed a difference in the shape of the colonies growing on the media contained plant extracts, which indicates the effect of aqueous and alcoholic extracts of saffron, cloves, black seed and miswak on the growth of *Candida* species.

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1. Introduction

Causes of disease in human and that threaten to cause invasive infections, the species causes diseases like the oral, esophageal, vulva, and internal parasites, as well as candidiasis that is invasive [1]. *Candida* types are opportunistic pathogens that are present in the normal flora of the mouth, but can cause severe infections when the immune system is diminished, such as in patients with Thalassemia (TM), *Candida albicans* is mentioned as the most marginalized type, both in individuals with health and TM. [2]. Denture stomatitis, also called atrophic candidiasis, is the most common fungal disease in elderly patients and those who wear dentures [3]. Because of the random and informed use of antibiotics, this has resulted in the emergence of strains that are more resistant to antibiotics than the original strain [4]. The increased tolerance to the most commonly employed fungicides with an anti-fungal component, including fluconazole and ketoconazole is generally observed with these drugs are *C. albicans* infections [5]. The utilization of phytotherapeutics defines a commendable approach to addressing resistant microbes, this is attributed to their capacity to target the major mechanisms of resistance to drugs, including efflux pumps, biofilms, and cell membranes, among others [6]. The screening of plants as a source is now being carried out worldwide for alternative antimicrobial medicines, due to Active compounds such as, phenols, quinones, flavonoids, alkaloids, terpenoids, tannins, lignans, essential oils, and certain secondary metabolites which are considered as antimicrobial and antifungals properties in plants [7]. This study was aimed to use plants extracts such as Black seeds (*Nigella sativa,* Saffron (*Crocus sativus* L), Cloves (*Syzygium aromaticum*), Miswak (*Salvadora persica L*) as alternative antifungals could be used in oral candidiasis.

2. Materials and Methods

Samples collection: Fifty-two samples of oral swabs were gathered from patients (male and female) with age average equal to 25.5 with different levels of clinical improvement (thrashing= 18, thalassemia = 15, orthodontics= 19). Clinical demonstrations were led by doctors who are specialized in the field, the samples were obtained from Diyala General Hospital and some health centers in Diyala province\Iraq. The sampling method involves gently stroking a sterile cotton swab over the moth's tissue (the tongue and roof), this is then divided into two samples: one will be examined immediately under a microscope in order to identify the presence of *Candida* cells that are budding, the other will be cultured on SDA medium.

Identification of Candida Isolates: *Candida* was identified depending on the morphological features on sabouraud dextrose agar at 37ºC for 24-48hrs and germ tube formation according to Kumar (2010) (8) to detect *Candida albicans* from non *albicans* species.

Germ tube formation (GT): The creation of germ tubes in the yeast's isolate was evaluated by combining a small portion of a isolated colony in 0.5 ml of human serum. This suspension was held at 37°C for 2.5 hours. The incubation period must be no longer than 3 hours as other species of yeast can begin to form germ tubes. A decrease in the incubated serum's concentration was placed on a slide, which was covered by a slide and observed by the microscope to see if it had germ tubes. The identification of *Candida* spp. is accomplished using

Chromogenic agar *Candida*: The morphologically distinct isolates were inoculated in CHROM agar *Candida* and incubated at (32°C for 72 hours), for the presumptive distinction of Candida species via the color that is developed by the colonies, as a result of the development of enzyme activity [9].

Antifungals Susceptibility Test: The isolates were activated by using a modified new process in this study which give good results; the activation saline is prepared by adding (9gm) of NaCl and (2 gm) dextrose to 100 distill water then sterilize the solution by autoclave, let the solution cool then add 5ml in clean test tube and inoculate it with yeast colony, incubate at 37 c for 3 hours. Then the activated isolates were cultured on Mueller-Hinton plates, the antifungal discs were placed on the inoculated plate, the plates were incubated within (30mins) for 18-24h at 37ºC in an inverted position. After incubation the diameters of the complete zone of inhibition were noted and measured in millimeters by the aid of a metric ruler.

Collection of plants used in the study: The plants used (saffron, cloves, black seed and miswak) were obtained from local markets in Baqubah, Diyala, Iraq and transported to the laboratory. The plants were ground to obtain a fine dry powder. The powder was kept in sterile and tightly closed boxes.

Preparation of aqueous and alcoholic plants extracts: According to the method of Ahmed *et al.* (1998) [10] aqueous extracts were prepared by mixing 20 g of the grounded plants used in the current study for each sample separately with 400 ml of distilled water in a 1000 ml volumetric flask, then leaving the suspension in a shaking water bath at a temperature of 40 ºC for 24 hours, then filtering the suspension using sterile filter paper. The clear liquid was stored in tightly sealed containers in the refrigerator at a temperature of 4°C until use. Alcoholic extracts were prepared. 95% ethyl alcohol was chosen to prepare the alcoholic extracts in the same way as preparing the aqueous extract, replacing the distilled water with ethyl alcohol.

Preparing culture media containing plant extracts: After preparing the extracts, the culture media was prepared as described in the method for preparing the media above. The media containing the extracts was prepared by adding plants extracts to the medium at two concentrations of 5 ml and 10 ml. 5 ml was mixed in 100 ml of the culture medium and 10 ml of the extracts were mixed with 100 ml of the culture medium. The media was sterilized and left to solidify at room temperature. Control media were prepared for the purpose of comparison to determine the effect of the alcoholic extract by adding 5 ml of ethyl alcohol to 100 ml of sterile culture medium, and the other concentration was 10 ml of ethyl alcohol added to 100 ml of culture medium to determine the extent of the effect of alcohol on the culture, apart from the effect of the plant extract itself.

Inoculation of culture media: The culture media containing the plant extracts were inoculated with the yeast suspension prepared in advance by taking 100 microliters of the yeast suspension into the culture medium and spreading it over the medium using a sterile cotton swab. The cultured dishes were incubated at 37°C for 24 hours.

Statistical analysis: Data of current study were analyzed by using Chi-square (X2) test to compared between percentages. Least significant different (LSD) used to compared among means. A level of significance of α=0.05 was applied to test. (SPSS v.22 and Excel 2013) programs were used to analyze current data.

3. Results and Discussion

Samples Collection: The patients involved in this study were segregated into three primary categories: Premature Baby's oral Thrash, Orthodontics, and Thalassemia.

The Results of Oral Specimens Examined by Direct Examination and Culture on SDA: The laboratory examination results showed that; 34 (65.38%) out of 52 oral swabs specimens; were positive for candida growth while 18 (34.61%) out of 52 specimens were gave negative results for *Candida* Spp. by direct examination on (10%) KOH and cultured on SDA. 13 (86.7%) out of 15 oral swab from thalassemia patients were positive for *Candida* spp. two (13.3%) were negative. Out of 18 premature babies' oral swab; 11 (61.1%) were positive for *Candida* spp. and 7 (38.9%) were negative. Whereas 10 (52.6%) out of 19 orthodontics oral swab were positive and 9 (47.4%) were negative for *Candida* spp. (figure 1).

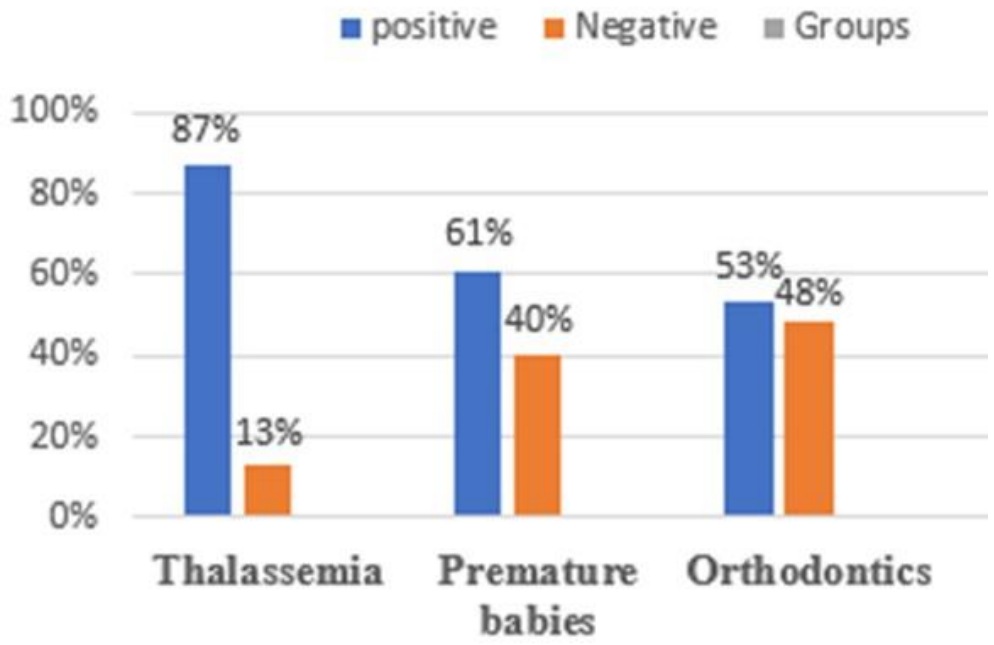

Figure 1: Positive and negative results of *Candida* spp.

Among study groups, statistical analysis test showed that; there were very high significant different ($p<0.001$) between positive and negative results of *Candida* growth among thalassemia patients group and significant differences ($p<0.05$) as total results (Table 1). Among thalassemia group this study found that *Candida* growth is too high comparing with the results of Karayilmaz *et al.* (2019) who found Less *Candida* was isolated in β-TM patients [11]. These results may cause, due to the iron overload in patients with beta thalassemia major, damage to the salivary glands leading to higher risk of dental caries, gingivitis, and *Candida albicans* infection [12]. Our results are closely related with Grzegocka *et al.* (2020) were reported that (58.8% of orthodontics users were *Candida*-carriers and the study concluded that orthodontic appliances promote *Candida* yeast colonization, which is variable over time in terms of strain and species [13]. conventional brackets are often used during orthodontic therapy of patients with malocclusion. The complex construction of such brackets greatly inhibits oral hygiene, which predisposes to increased carriage of bacteria and yeasts [14, 15]. Casaccia *et al.* (2007) mentioned that elastomeric rings are one element of conventional orthodontic brackets that used to bind the bracket with the orthodontic wires, these rings have rough and irregular surface which improve the colonization of microorganisms [16].

Table 1. Distribution of *Candida* growth among study groups are calculated by chi-square Test:

	Candida spp.			
	Positive	Negative	Total	P value
Thalassemia	13 86.7%	2 13.3%	15	0.001***
Premature babies	11 61.1%	7 38.9%	18	0.345
Orthodontics	10 52.6%	9 47.4%	19	0.818
Total	34 (65%)	18 (35%)	52	0.021*
P value	0.763	0.196	0.587	
*= significant different (p<0.05) ***= very highly significant different (p<0.001)				

Identification of *Candida* spp. by *Candida* chromogenic Agar Medium: Candida colonies that were cultivated on SDA were then subcultured on media that produced colors. The isolated organisms were able to grow and develop colonies that were distinctively colored after being incubated for 12 hours. Presumptive identification was accomplished by noting the color of the colonies according to the manufacturer's instructions (*C. albicans* - green, *C. tropicalis* - blue, *C. krusei* - pink colonies that have a matt surface, *C. dubliniensis* - green, *C. glabrata* - Pale cream (figure 2). In this study, from 34 *Candida* isolates from oral swabs; 19 (56 %) were *C. albicans* and 15 (44%) were non albicans (Table 2). The current results show that: *C. albicans* was the most predominant spp. among different specimen's groups fallowed by *C. trupicalis, C. glabrata* and lastly *C. krusei*. with high significant different. These findings are associated with Bhattacharjee [17] who documented that of the 70 samples, *C. albicans* was isolated from 34 (48.57%) samples.

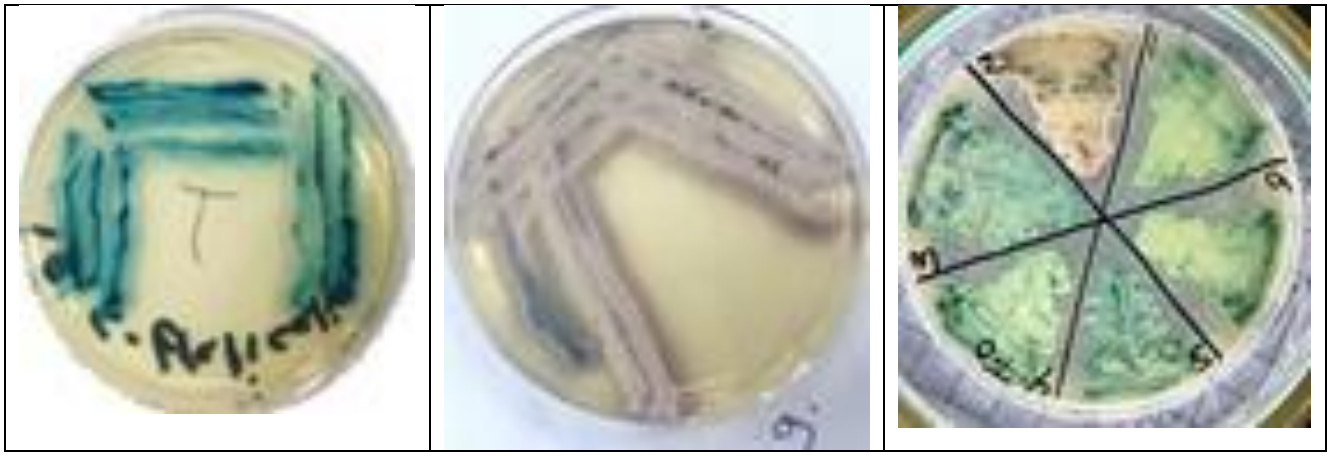

Figure 2. The green colonies of Candida albicans ,11-pink to purple colonies of C. krusei: blue growth of C. trupicalis; pale for C. glabrata on chromogenic agar medium 37 c/ 24 h.

Table 2: Distribution of *Candida Spp.* among study groups.

Specimen	*Candida Spp.*				Total number	P value
	C. albicans	*C. trupicalis*	*C. glabrata*	*C. krusei*		
thalassemia	9 (69.23%)	3 (23.07%)	0	1 (7.7%)	13	0.001***
Premature babies	6 (54.54%)	4 (36.36%)	0	1 (9.1%)	11	0.19
orthodontics	4 (40%)	4 (40%)	2 (20%)		10	0.66
Total	19 (56 %)	11 (32%)	2 (6%)	2 (6%)	34	0.004**
P value	0.35	0.19	1.00	0.63		
**= high significant different (p< 0.001)						

The remaining 36 (51.43%) were non-albicans *Candida* (NAC). Mohammed et al. [18] documented that in candidiasis that occurred in the oral cavity; *C. albicans* was the most common species (47%). Among the Thalassemic samples, the current results are essentially identical to El-Kasem's. [19] that *C. albicans* were documented in 69.2% of instances, while 30.8% were not. *C. albicans.* (23% *C. tropicalis* and 7.8% *C. krusei*). C. *albicans* was frequently re-covered from healthy children (57%) and *C. albicans* is the most common isolated species from the oral cavities of patients with thalassemia [12]. The possible cause of the increased rate of yeast in the blood of patients with thalassemia is that in the absence of or insufficient chelation therapy, there is an increase in the accumulation of iron in the blood that is not transferred to the cells, free iron, and ineffective erythropoiesis [20]. Our results concluded that *Candida* spp are most prevalence in thalassemia patients fallowed by premature babies and orthodontics oral swabs, the reason of this prevalence that the low immunity in thalassemia patients and premature babies in addition to the probability of contaminated materials and milk bottles that used in babies feeding. In the other hand; orthodontic that used to correct teeth make the cleaning and brushing teeth more difficulty and the brash teeth cannot reach to the covered area that caused the remaining of food residues which provide sugar source for *Candida* overgrowth.

Antifungal Susceptibility Test: Eight *Candida* isolate (two from each detected species) were chosen to determine the antifungal susceptibility test. Three antifungals were used in this study Fluconazole (25 µg), Clutrimazole (50 µg) and Posaconazole (5 µg), Colestin an antibacterial was used as a control. Disc diffusion method was used by adding antifungal discs to Molar Hinton agar plates inoculated with *Candida* inoculum. The current results show that *C. albicans* isolates were the most affected by Clutrimazole at concentration 50 µg with 4 cm and 3.9 cm inhibition zone (table 3), followed by Fluconazole and posaconazole *C. trupicalis* and *C. krusei* were resistant to Fluconazole at concentration 25 µg (figure 3) , was the last affected by antifungals were used in this study (2 cm inhibition zone for clotrimazole, 0.3, 0.8 cm for fluconazole and posaconazole respectively) with least significant differences (0.7). Resistance typically occurs as a result of mutation in the sterol synthesis pathway, this is also true of Clotrimazole and Fluconazole, which are both members of the class of anti-fungal agents. The polyene antifungal agents include nystatin, amphotericin B, and pimaricin [21]. The widespread use of these drugs for prophylaxis in these patients led to the colonization of *Candida* species and a resistance to these drugs [22].

Table 3: The inhibition zone of antifungals used in this study

Isolate No.	*Candida* Spp.	Clo (50 µg)	Flu (25 µg)	Pps (5 µg)	Cs	LSD
1	*C. krusei*	2 cm	0.3 cm	0.8 cm	0.0	0.7
2	*C.krusei*	1.9	R	R	R	NS
3	*C. trupicalis*	2.1	R	0.3(R)	R	0.99
4	*C. trupicalis*	3 (S)	2	2.5(S)	R	0.8
5	*C. glabrata*	3(S)	3.5(S)	3(S)	R	NS
6	*C. glabeata*	2.5 (S)	2.5	2.5	R	0.94
7	*C. albican*	4 (S)	3.9(S)	3(S)	R	NS
8	*C. albican*	3.9 (S)	3.6(S)	3(S)	R	
LSD		1.11	1.29	2.09		

Clo: Clutrimazole (50 µg), **Flu**: Fluconazole (25 µg), **Pps:** Posaconazole (5 µg), **Cs**: Colestin, **R**: resistant **S:** sensitive, LSD: least significant differences, NS: non-significant.

Figure 3. Antifungals inhibition zone of 1- *C. trupicalis* 2- *C. krusie* on Moler Hinton agar at 37 c for 24h.

The effect of plant extracts on the growth of *Candida* yeast. The results of cultivation on media poisoned with plant extracts showed an unnoticeable variation in yeast growth, the four types of *Candida* yeast were able to grow on poisoned media at both concentrations of 5 ml and 10 ml of the plant extracts used. The culture medium poisoned with the alcoholic extract of saffron (*Crocus sativus*) (Figure 4) showed a clear effect against the yeast growth of *Candida* species to a greater degree than the aqueous extract of the same plant, as it showed greater growth in terms of the number of colonies and colony size. The medical and therapeutic effects of saffron are due to various compounds such as crocin, crocitin and flavonoids these compounds have therapeutic activities particularly crocin which act as anti-inflammatory, anticancer as well as analgesic [23].

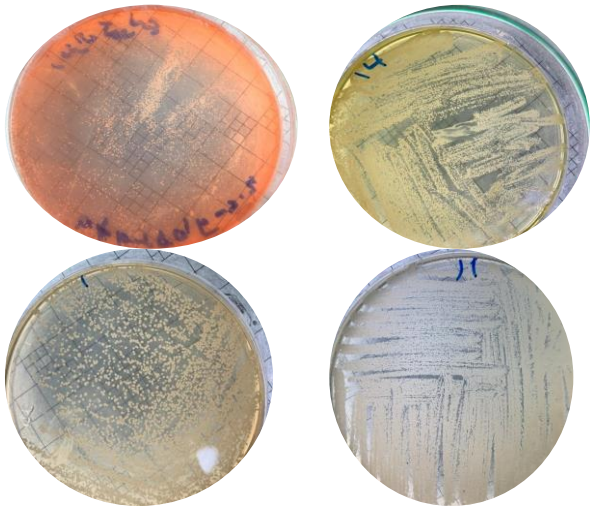

Figure 4. 1-*Candida* growth on alcoholic extract of saffron 2-*Candida* growth on aqueous extract of saffron 3-*Candida* growth on aqueous extract of cloves 4-*Candida* growth on aqueous extract of black seed.

Aslani *et al.* [24] reported that the high MIC value of Crocin for Candida species indicated that it failed to demonstrate effective antifungal activity against this species. The MIC of this substance was not reduced significantly by the addition of fluconazole, even though the latter was present in a concentration of 0.3%, this suggests that the aqueous extract of saffron has a limited effect on *Candida* in this study, in contrast, the alcoholic extract is effective against this bacterium and this is in agreement with previous studies that employed the petroleum ether and methanolic extracts of saffron flower. These compounds demonstrated a strong capacity to inhibit bacteria and fungi [25, 26]. Karbasaki *et al.* [27] discovered that saffron possessed antifungal abilities against *C albicans*. As a result, saffron could be considered a beneficial ingredient for use in mouthwashes, the herb's origin is herbal, the cost is effective, and there are fewer adverse effects. The aqueous extract of cloves and the miswak showed a somewhat similar effect on yeast growth, and the aqueous extract of cloves had a greater effect than the aqueous extract of saffron, which indicates the efficiency of cloves in reducing excessive growth of *Candida* isolated from the mouth because of its Oral healing properties. *Salvadora persica* is often referred to as Miswak in Islamic cultures, it is part of the Salvadoraceae family and is used as a stick to chew or a toothbrush [28], The antimicrobial and cleansing effects of miswak are attributed to various chemical compounds in its extracts, including sodium chloride and potassium chloride, as well as salvadourea and Salvadorine, saponins, tannins, vitamin C, silica, and resin [29]. Significant antibacterial and antifungal activity of *S. persica* has been documented [30]. The presence of benzyl isothiocyanate in this plant is believed to be crucial to avoid the formation of acid and the growth of bacteria. It possesses additional properties that inhibit the reproduction of viruses and prevent the

development of fungal diseases [31]. *Syzygium aromaticum*, also known as Cloves, are dried flowers with an aromatic nature that are exposed by the Myrtaceae family, these flowers are utilized as seasonings around the world and their oil have two significant components, eugenol and ß-caryophyllene [32]. The primary component of clove oil, eugenol (2 methoxy-4 allyl Phenol), may be responsible for the activity of clove in antibacterial, antifungal, and tannin properties, as well as the high content of tannin (10-19 %) [33]. Shareef *et al.* [34] divulged that the extracts from *S. aromaticum* and *S. persica* have antibacterial and antifungal properties that are directed against both gram positive and negative bacteria, as well as fungi. Aqueous and alcoholic extracts of miswak were effective against *C. albicans,* the greatest effectiveness was achieved with a concentration of 200 mg/ml of aqueous extract [35]. The current study found that the aqueous extract of black seed (*Nigella sativa*) or can called Habat barakah locally, did not show a clear effect on the growth of *Candida* yeast, as the growth was very good (figure 4), while the alcoholic extract showed a slight effect on growth, these results are agreed with Ariamanesh *et al.* [36] that *N. sativa* extract has week effect on *Candida* comparing with nystatin due to the indirect immune-regulatory effects of *N. sativa*. Darmawan *et al.* [37] mentioned that *N. sativa* has antifungal effects against *C. albicans*, the antifungal activity of *N. sativa* is due to the components of thymoquinone, thymol, and carvacrol, and the optical density of *C. albicans* incrementally decreased on exposure to increasing concentrations of *N. sativa*. The current study showed that the alcoholic extract of saffron has the most effect on the yeast growth of *Candida*, followed by the aqueous extract of cloves. While the rest of the plant extracts showed a lesser effect on growth, especially the aqueous extracts, while the alcoholic extracts were more effective to varying degrees. The variation in the effectiveness of extracts against fungi may be due to the difference in the polarity of the solvents used in the extraction, a difference that affects the solubility of some of the active substances present in the plant. The superiority of the alcoholic extract over the acetone and aqueous extract in inhibiting the growth of fungal isolates may be due to the ability of ethyl alcohol to dissolve many active substances dissolved in alcohol and in other polar and nonpolar solvents [38]. The reason for the high values of the minimum inhibitory concentration for other plant extracts can be attributed to the presence of some active compounds in these plants, but in low concentrations [39]. Since the conditions and method of extraction are uniform, as well as the conditions of tests for antagonistic activity against fungi, this difference in values and rather the superiority of the alcoholic extract in terms of lower minimum inhibitory concentration values can be attributed to its polarity, which plays a major role in the extraction of some active compounds, depending on the plant species used [40] .This study also found that these extracts of the plants under study had clear effects on the size and shape of the colony on the dish, regardless of the number of colonies inside the dish, which was attributed to a number of reasons, including the large amount of inoculum used, as it was not compared to the standard McFarland measurement methods. The number of cells taken during inoculation of culture media contaminated with extracts was not known, which was a major reason for the inaccuracy of measuring the extent of the effect of plant extracts on yeast. As for the effects of the aforementioned plant extracts, they were represented by the small size of the colony on the culture medium to a very clear extent, until it was believed that it was bacterial contamination (the size of a pin head), as it was not possible to confirm that it was yeast by looking at the shape of the colony only, and reliance was placed on microscopic examination and observation of the budding cells. Microscopic examination showed that the shape and size of the single cell were also affected due to its growth on media containing plant extracts, as the yeast cell appeared spherical instead of being elongated oval in the normal state. The budding process was also observed to be affected, and pseudo hyphae were not observed in any isolate, which can be observed. In the case of growing *Candida* yeasts on the culture medium Sabouraud Dextrose Agar, which proves the effect of the aqueous and alcoholic extracts of the plants used in the study to varying degrees.

4. Conclusions

This study concluds that orthodontics promotes the growth of Candida species in addition to that many types of Candida were isolated from thalassemia patients and *C, albicans* was the predominant species among premature babies, this study finds that both the aqueous and alcoholic extracts of the chosen plants were showed antifungal effects on *Candida albicans* growth which make them as good choices to use these plants as alternative natural treatments for oral candidiasis.

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Conflict of Interest
The authors declare that there are no conflicts of interest regarding the publication of this manuscript

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