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Detection of Oral Candida Species and Investigation from Anti-Candida Activity of *Origanum vulgare* L. Essential Oil

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Abstract: Oral candidiasis is mainly caused by the yeasts in the normal flora of the oral cavity, especially the frequency of life-threatening infections caused by pathogenic Candida species has increased worldwide. The aim of this study was to identify oral Candida species and evaluated the anti-Candida activity of essential oil obtained from *Origanum vulgare* L. Identification of 49 of 60 (81.6%) Candida isolates from children patients at Malaysia were determined by classical identification methods. The most frequently isolated species of yeast from oral swabs was *Candida albicans*, which accounted for 24 (48.9%) of the total isolates followed by *Candida parapsilosis* 12 (24.4%), *Candida tropicalis* 9 (18.3%) and *Candida glabrata* 4 (8.1%). The essential oil of *Origanum vulgare* L. was prepared by hydro distillation using a Clevenger apparatus and eighteenth compounds of the essential oil of *O.vulgare* L. were identified by gas chromatography (GC) using flame ionization (FID) and gas chromatography coupled mass spectrometry (GC/MS), the major components were Carvacrol (20.04%), Thymol (7.45%), P-cymene (6.62%), β-caryophyllene (5.5%) and α-terpinene (5.4%). In addition, the study examined the essential oils of *O.vulgare* L. against isolated Candida species by an agar well diffusion method and serial broth dilution method. Finally, the results showed that essential oil from *O.vulgare* L. exhibit strong antifungal activity than Nystatin antibiotic on all isolated Candida species, the lower minimum inhibitory concentration MIC was observed for essential oil (0.6 µl/ml) for *C.tropicals* and *C.albicans* comparing with the MIC for Nystatin (32 µg/ml).

Key words: Oral Candidiasis · Anti-Candida Activity · *Origanum vulgare* L.

INTRODUCTION

Yeasts are opportunistic pathogens and common members of the normal flora in humans. Such infections may either be superficial, affecting the skin, hair, nails and mucosal membranes, or systemic involving major body organs [1]. A number of factors have been implicated in this increased occurrence of fungal disease, but it is generally accepted that the increased and widespread use of certain medical practices, such as immunosuppressive therapies, invasive surgical procedures and use of broad-spectrum antibiotics are significant [2, 3]. Oral candidiasis is a common opportunistic infection of the oral cavity caused by an overgrowth of Candida species, which appears to be ulcers develop to become white spots. Infection is called oral thrush, it is appear in new infants due to low PH of saliva, or due to transferred infection from pregnant infected mother to the new infants during delivery [4]. *Candida albicans* is the most commonly isolated yeast species in the oral cavity both in health and disease. It accounts for about 47% to 75% of the oral yeast isolates, while other medically important yeast pathogens *Candida tropicalis*, *Candida parapsilosis*, *Candida krusei*, *Candida kefyr*, *Candida glabrata* and *Candida guilliermondii* each represent less than 10% of isolates [5]. The most frequently used classification to describe various types of oral candidiasis is by Lehner [6]. The categories in Lehner's classification include acute pseudomembranous candidiasis, acute atrophic candidiasis, chronic atrophic candidiasis and chronic hyperplastic candidiasis. The latter comprises chronic oral candidiasis, endocrine candidiasis syndrome, chronic localized muco-cutaneous candidiasis and chronic diffuse candidiasis.

Several factors, such as virulence of the infecting yeast strains (such as adhesions, rapid phenotypic switching, hyphal growth and secretion of hydrolytic enzymes), host resistance and various environmental

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factors, including sugars and antibacterial drugs, influence risk for the development of candidiasis [7]. For treatment of fungal infection; many antifungal have been used such as compounds of polyenes and azoles [8]. Due to the increasing development of drug resistance in human pathogens as well as the appearance of undesirable effect of certain antimicrobial agents, there is a need to search for new antifungal agents without toxicity and side effects. Therefore, it is necessary to search for more effective and less toxic novel antifungal agents that would overcome these disadvantages, traditional medicine derived from plants are still being used in different parts of the world.

Origanum vulgare L. is used all over the world as a spice and medicinal plants. It is a perennial herb from the family Lamiaceae; it is native to the Mediterranean and Eurasia and grows in mountainous areas with rocky, calcareous soil. *Origanum* is known widely in the world it's rich in the phenolic monoterpenoids, mainly carvacrol, occasionally thymol. A number of chemical compounds i.e. α-terpinene, p-cymene, p-cymen-8-ol, α-thujene, sabineneare also present. Two more biochemical groups, usually of less significance quantitatively, are present in origanum: that of the acyclic monoterpenoids such as geraniol, linalool and β-myrcene and that of bornane type compounds such as camphene, camphor and bornyl. To the above groups of compounds some sesquiterpenoids, such as β-caryophyllene, germacrene, α-cadinene, α-copaene, α-cadinol, β-caryophyllene oxide and germacrene-D-4-ol should also be added [9]. The most bioactive compounds in essential oils of *O.vulgare* L. are the phenols carvacrol and thymol. These presumptions are in accordance with the findings of Hussain [10], who reported that essential oil of *O.vulgare* (rich in carvacrol and thymol) showed potent antimicrobial activity. Other authors documented the inhibitory effect of *Origanum* essential oil showed potent against *Candida albicans* [11, 12]. The goal of this study is to identify Candida spp. and also the anti-Candida activity of *Origanum vulgare* L. essential oil has been evaluated.

MATERIALS AND METHODS

Isolation and Identification of Candida spp. From Patients: Oral swabs were collected from 60 children aged between 1 day-10 years and suffering from oral thrush. All specimens were collected from General Hospital Pulau Pinang at Malaysia from January 2013 until March 2014. The specimens were taken by sterilized cotton swabs and were divided in to two smears: one smear was examined immediately under microscope for direct examination, the other usually was transferred to the laboratory for culturing the swabs were cultured on CHROMagar Candida and incubated at 30°C for 24-48 h. Presumptive identification of isolates was attempted on the basis of the characteristic coloration of the colonies on CHROMagar Candida. All isolates were purified by sub culturing on Malt extract agar MEA and identified to the species level using API 20C AUX Kit. The isolates suspected to be *C.albicans* were also studied for germ tube formation in human serum incubated at 37°C for 2 h [13-15].

Plant Material Collection and Isolation of their Essential Oil: The leaves of *Origanum vulgare* L. were obtained from Jordan. Plant was identified and authenticated by plant taxonomist, then kept in a cool dry place until ready for extraction of the essential oil. Afterwards, essential oil was taken from 50 g of the dried leaves of *O.vulgare* in hydro distillation method for 3 h using a Clevenger type apparatus, following the sample oils were dried with anhydrous sodium sulfate and kept in sterile sample tubes in refrigerator until further tests; the process was repeated several times to collect enough of the essential oil for further analysis the essential oil and studied the biological activities of essential oil.

Analysis of Essential Oil

Gas Chromatography (GC) and Gas Chromatography/Mass Spectrometry (GC/MS): The quantitative analysis was done by GC HP-5890 II apparatus, equipped with a split/splitless injector, attached to an HP-5 column (25 m × 0.32 mm, 0.5 µm film thickness) and fitted to a flame ionization detector (FID). Operating conditions were as follows: Carrier gas used was ultra pure nitrogen degree at a flow rate of 1 ml/min, split ratio 1:30.Column temperature was held constant at 40°C for 10 min, then heated to 280°C at 5°C/min and held constant at 280°C for 10 min, injector temperature was 260°C. The volume of injected specimen was 0.5 µl of diluted oil in hexane (1:10). The same analytical conditions were employed for GC/MS analysis, where HP G 1800C Series II GCD system equipped with an HP-5MS column (30 m × 0.25 mm, 0.25 µm film thickness) was used and the transfer line was heated at 280°C. Mass spectra were acquired in EI mode (70 eV), in an m/z range of 40-400. The gas vector (Helium) was fixed to 1.5 ml/min the volume of injected specimen was 1.5 µl of diluted oil in hexane. Electron impact identification of individual

constituents was made by comparison of their retention times with those of analytical standards of available terpenoids and by computer searching, matching mass spectra with those held in the Wiley 275 library of mass spectra. Confirmation was done using a calibrated AMDIS program for the determination of experimental values for retention indices of recorded constituents and comparing them with those from the literature [16]. For quantification purpose, area percent data obtained by FID were used.

Biological Activities of Essential Oil

Agar well Diffusion Method: The antifungal activity of the essential oil was determined by agar diffusion method [17]. Yeast suspension was prepared in concentration 1.5×10^8 cells/ml by suspending one isolated colony from Malt Extract Agar (MEA)in 10 ml of Malt Extract Broth (MEB) and overnight broth cultures at 28-30°C, then 0.1 ml of suspension was transferred to MEA and spreading by glass spreader and calculated the cell. Essential oils were diluted in Dimethyl Sulfoxide (DMSO) to different concentrations (10, 50, 100, 300 and 500 µl/ml), 0.2 ml was taken of Candida spp. suspension 10^8 cells/ml was put on surface of MEA media and spread by using glass spreader L-shaped, left to dry for 10-20 minutes, then by using sterilized cork borer 8 mm diameter, wells were made in the MEA medium then add 0.1 ml for each concentrations of the plant extract in three repeated Petri dishes for each treatment and incubated at 30°C for 24-48 h, DMSO was used as control. The activity of each concentration of the different extracts was determined by measuring of diameter of the inhibition zone around each well using the ruler.

Serial Dilution Method: Minimum Inhibitory Concentrations (MICs) and Minimum Fungicidal Concentrations (MFCs) of essential oil were determined based on a micro-dilution broth method in 96-well plates as previously described [18]. The suspension of Candida strains were prepared from liquid culture and adjusted to a final density of 10^6 cells/ml and used as an inoculum. Essential oil was dissolved in DMSO and then in Muller Hinton Broth (MHB) to reach a final concentration of 200 µl/ml. Then the 96-well plate was used for determination of MIC. After that, 95 µl of MHB and 5 µl of microbial suspension were added to every well, 100 µl of the essential oil with concentration of 200 µl/ml was added to the first well. Then 100 µl was taken from the first well and it was transferred to the next well. This process went on to the 7th well, serial twofold dilutions were made in a concentration range from 20, 10, 5, 2.5, 1.25, 0.6 and 0.3 µl/ml, the last well was contained 195 µl of MHB culture medium and also 5 µl of microbial suspension without any essential oil, this well was considered as negative control, then it was put in an incubator for 24 h in an appropriate temperature 28-30°C. In well plate, a column with antifungal was used as positive control (Nystatin in serial dilutions 128-0.125 µg/ml). To determine the MIC and MFC, the suspensions (20 µl) were taken from each well without visible growth and inoculated in Muller Hinton agar (MHA) for 24 h at 30°C. The MIC was defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth. The MFC was defined as the lowest concentration of the essential oil at which incubated microorganisms are completely killed. Last but not least, it should be pointed out that the data were presented as means ± Standard Deviation (SD). A computer program (SPSS version 10) was used for statistical analysis. The one-way ANOVA and Duncan test were performed to examine the differences among the groups.

RESULTS

A total of 60 hospitalized children swabs, which 49 patients (81.6%) had positive for oral thrush who were identified by The conventional methods of identifying yeasts to the species level in the clinical microbiology laboratory rely on criteria. The range of ages was classified to groups of 1-6, 6-12, 12-24 and 36-48 months. The highest infection incidence was seen in the age group of 1-6 months is 40.8%, while the ratio of infection was decrease in the age group of 36-48 months is 8.1%. There are high significant differences in distribution between age groups as shown in Figure 1.

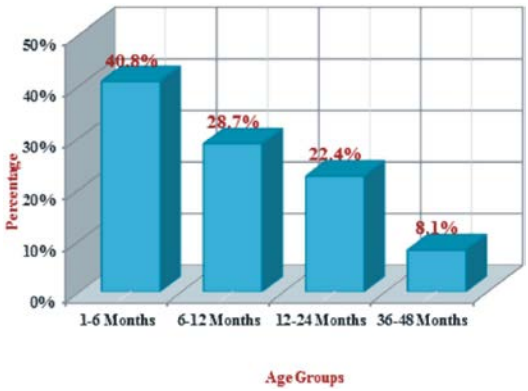

Fig. 1: Distribution of oral thrush in children patients according to age groups

Table 1: Morphological characteristics of Candida species

Species	Germ Tube Production	Hyphae/Pseudohyphae	CHROMagar Colony Colour
C. albicans	+	+/+	Blue-Green
C. parapsilosis	-	-/+	White
C. tropicalis	-	± /+	Dark Blue
C. glabrata	-	-/-	Pink-Purple

Table 2: API 20C AUX results for Candida spp

Identity	RAF	MLZ	TRE	SAC	MAL	LAC	CEL	NAG	MDG	SOR	INO	GAL	XLT	ADO	XYL	ARA	2KG	GLY	GLU
C.albicans	-	-	-	+	+	-	-	+	-	-	-	+	-	-	+	-	+	-	+
C.parapsilosis	-	+	+	+	+	-	-	+	+	-	-	+	+	+	+	-	+	+	+
C.tropicalis	-	-	+	+	+	-	-	+	-	+	-	+	-	-	+	-	+	-	+
C.glabrata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

where GLU = D-glucose,GLY = glycerol, 2KG = calcium 2-keto-gluconate, ARA = L-arabinose, XYL = D-xylose, ADO = adonitol, XLT = xylitol, GAL = D-galactose, INO = inositol, SOR =sorbitol, MDG = methyl-α-D-glucopyranoside, NAG =N-acetyl-glucosamine, CEL = D-cellobiose, LAC =D-lactose, MAL =D-maltose, SAC = D-sucrose, TRE = D-trehalose, MLZ = D-melezitose, RAF= D-raffinose.

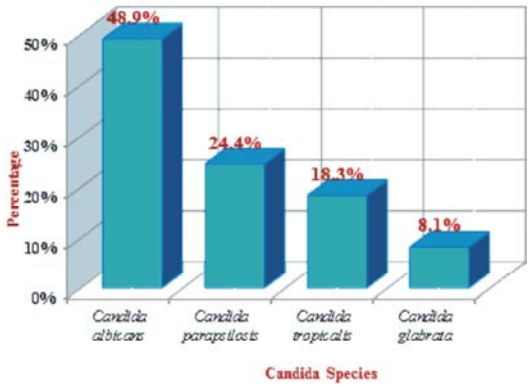

Fig. 2: Identification of different species for the Candida isolated by conventional method

Table 3: Chemical composition of essential oils of *Origanum vulgare* L

Component	Retention Index (RI)	Percentage (%)
α-Thujene	931	1.2
α-Pinene	939	0.5
Camphene	948	0.5
p-Cymene	976	6.62
Sabinene	983	1.5
α-Terpinene	998	5.4
Limonene	1018	4.9
1,8-Cineole	1039	0.3
Linalool	1082	2.42
Terpinolene	1088	0.6
Verbenol	1122	0.41
Carvone	1188	0.6
Thymol	1290	7.45
Carvacrol	1298	20.04
β-Caryophyllene	1418	5.5
Caryophyllene oxide	1506	1.50
α-Cadinol	1653	0.6
Carvone	1942	1.1
Total		61.1

The results of the morphology of Candida species colonies on Malt extract agar were white to cream, round, curved, soft and smooth to wrinkled and microscopic examining of Candida spp. isolates appeared gram positive, spherical to oval with the present of budding, while pseudohyphae appears after lactophenol cotton blue staining. Presumptive identification of isolates was attempted on the basis of the characteristic coloration of the colonies on CHROMagar Candida and the isolates suspected to be *C.albicans* were also studied for germ tube formation in human serum incubated at 37°C for 2 h. Identified to the species level were confirmed by commercial sugar assimilation tests (API 20C AUX Kit) as shown in Tables 1 and 2.

From this study four species were obtained of Candida. *Candida albicans* was the major species accounting for 24 (48.9%) of the total isolates. The next most frequent Candida species were *Candida parapsilosis*, which was isolated from 12 (24.4%). Other species less ratio were *Candida tropicalis* 9 (18.3%) and *Candida glabrata* 4 (8.1%) as shown in Figure 2.

Moreover, by using gas chromatography-mass spectrometry were obtained of the eighteenth volatiles constituents in the essential oil of *Origanum vulgare* (Table 3). The major components were identified as carvacrol (20.04%) and thymol (7.45%), while p-cymene, β-caryophyllene and α-terpinene were also dominant constituents with slightly lower percentage (6.62%, 5.5% and 5.4%). The other components were less than 5%.

Preliminary screening of the in vitro antifungal activity of the essential oil of *Origanum vulgare* extract against four pathogens microorganisms was studied using an agar well diffusion method. The diameters of the inhibition zones are presented in Table 4. The results showed that the isolates sensitivity was increased with the increase of antifungal concentration ($p<0.05$). In the isolates of Candida spp. gave the highest inhibition zone between (50-52.67 mm) in the concentration (500 µl/ml), whereas it's gave (9.67-12 mm) in diameter of inhibition zone with the concentration (10 µl/ml). It was found that

Table 4: Anti-Candida activity of *Origanum vulgare* L. essential oil by using an agar diffusion method

	Inhibition Zones Diameter (mm)					
	Concentration µl/ml					
Species	DOMSO	10	50	100	300	500
C. albicans	0±0[a]	11±1[b]	28±1.45[c]	36.33±1.53[d]	48.67±1.58[e]	52±0.58[f]
C. parapsilosis	0±0[a]	9.67±0.58[b]	24.67±1.53[c]	34.33±1.04[d]	47.33±0.58[e]	50±1[f]
C. tropicalis	0±0[a]	12±1[b]	29.67±0.58[c]	35.45±0.5[d]	49±1.65[e]	52.67±0.53[f]
C. glabrata	0±0[a]	10±0.36[b]	24.67±1.55[c]	31.67±1.08[d]	50.1±0.65[e]	51±1.53[e]

Values represent mean ± standard deviation of triplicates.

Means followed by different superscript letters in the same row represent significant difference ($p<0.05$).

Table 5: Minimum Inhibitory Concentrations (MICs) and Minimum Fungicidal Concentrations (MFCs) of *Origanum vulgare* L. oil and Nystatin against Candida Species

	Origanum vulgare L.		Nystatin	
Candida Species	MIC (µl/ml)	MFC (µl/ml)	MIC (µg/ml)	MFC(µg/ml)
C. albicans	0.6	1.25	32	64
C. parapsilosis	1.25	2.5	32	64
C. tropicalis	0.6	1.25	32	64
C. glabrata	1.25	2.5	32	64

the essential oil of *Origanum vulgare* was a strong inhibitor against *C.tropicalis*, *C.albicans* and *C.glabrata* a moderate inhibitor, whereas the less inhibitor was against *C.parapsiolsis*. There were significant differences in isolates sensitivity to different antifungal concentrations ($p<0.05$).

The MIC and MFC to the essential oil of *O.vulgare* tested by serial dilution method and compared with Nystatin activity are displayed in Table 5. *C.tropicalis* and *C.albicans* were inhibited at lower concentrations MIC (0.6 µl/ml) and MFC (1.25 µl/ml), while *C.parapsilosis* and *C.glabrata* were the most resistant fungi towards this oil; MIC was 1.25 µl/ml and MFC 2.5 µl/ml and compared with Nystatin activity used as positive control, MIC and MFC value of antibiotics "Nystatin" were 32 µg/ml and 64 µg/ml respectively to all the Candida spp. have been isolated.

DISCUSSION

The laboratory examination results showed that 49 specimens from 60 oral swabs of Candida spp. were obtained in this study in percentage (81.6%). These results are close to other researchers' results in this field. Our results agree with the findings of Hassan [19] who found the ratio of infection (84.61%) have been isolated from leukemic children under chemotherapy [20] mentioned that Candida infection ratio was (95.59%) from patients with invasive candidiasis in Kuala Lumpur Hospital, Malaysia. Our study results are higher than the findings of Pignato *et al.* [21] mentioned the isolation rate is 63.0%, which isolated from oral carriage in Italian HIV-seropositive subjects. Besides that, Badiee and Alborzi [22] mentioned the isolation rate is 70%, which isolated from the immunocompromised patients in Iran.

In the present study, we found that candidiasis might be at all ages where the youngest is one month neonates while the oldest is 10 years. The majority of the patients were in the age group of 1-6 months, therefore, this might be related to the fact that the source of yeasts from mother to child by oral as well as Candida species can be transmitted to the oral cavity of the child from other body sites such as the mother's vagina during delivery [23, 24]. On the other side, the recovery of oral yeasts from these children was associated with use of a pacifier beyond age 12 months, eruption of the first teeth after 6 months of age, the mother cooling the child's food by blowing on it and the mother cleaning the child's pacifier in her own mouth [25]. According to study conducted by Hannula [25] found majority of children patients is 31% of 1-6 months, 20% at 12 and 18 months as well as 13% at 24 months. This disagrees with another Finnish study of 12-48 months whom the occurrence of yeasts was associated with antibiotics [26]. In our study, the most common species that has isolated of the children is *C.albicans* (48.9%). The *C.parapsilosis* (24.4%) showed

to be the second isolated Candida species from oral cavity, while the *C.tropicalis* and *C.glabrata* have been isolated a minor ratio (18.3% and 8.1% respectively). The Candida species ratios found agree with data reported of previous studies [20, 27] who found *C.albicans* is the most commonly isolated yeast species in the oral cavity additionally other medically important yeast pathogens *C.tropicalis*, *C.parapsilosis*, *C.krusei* and *C.glabrata*.

As for the *Origanum vulgare* L., we have been isolated and characterized the major chemical components of the essential oil of *O.vulgare* L. obtained by the GC-MS. The findings are carvacrol (20.04%) and thymol (7.45%), while other components were also dominant constituents with slightly lower percentage. These components are similar to the findings of study in Morocco which the major constituents were carvacrol (18.06%), thymol (7.36%) and p-Cymene (5.02%) [28]. On the contrary, in study at Lithuania, the components of β-caryophyllene (15.4-24.9%) was the main constituent in the oil of *O.vulgare* and contained low levels of phenols are thymol 0-3.2% and carvacrol 0-1.3% [29].

The antifungal effectiveness of essential oil for *Origanum vulgare* L. was evaluated by the measurement of Inhibition Zones (IZ), Minimum Inhibitory Concentration (MIC) and Minimum Fungicide Concentration (MFC). The tested essential oil of *Origanum* is inhibitory to the growth of all the Candida spp. that has been isolated.The results of the bioassays showed the two Candida Species are *C.tropicalis* and *C.albicans* high sensitivity against the essential oils, which have been diameters of inhibition zone ranging from 11-52.67 mm with the lowest MICs and MFCs are 0.6 and 1.25 µl/ml respectively. The activity is often attributed of essential oil of *Origanum vulgare* due to contain mainly aromatic monoterpenes, carvacrol, thymol, p-cymene, caryophyllene and α-terpinene. Several studies have demonstrated the antimicrobial activity the essential oils of many species of the genus *Origanum* rich in volatile phenols, the most of *Origanum* genus have been investigated for their antimicrobial properties against some bacteria and fungi. It is depending on thymol and carvacrol among their main constituents and these are accompanied by other compounds, which are present in lower concentration [28, 30, 31]. Finally, the action mechanism of phenolic compounds was related to the ability of phenolic compounds to alter microbial cell permeability, thereby permitting the loss of macromolecules from the cell interior, could help explain some of the antimicrobial activity. Another explanation might be that phenolic compounds interfere with membrane function and interact with membrane proteins, causing deformation in structure and functionality [32].

CONCLUSION

Two important results are concluded from this research about isolated Candida species and the essential oil activity of *Origanum vulgare* L. The most frequently isolated species of oral cavity are *Candida albicans* and *Candida parapsilosis* of 48.9% and 24.4% respectively. In addition, we found the essential oil of *O.vulgare* L. exhibit strong anti-Candida activity.

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