In Vitro: Comparative study of Inhibitory Activity of *Olea europaea subsp. cuspidate* Leaf Extracts Against Oral Candidiasis

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Abstracts: The present study aimed to investigate the potential inhibitory activities of (*Olea europaea subsp. cuspidate*) olive leaf crude extracts. HPLC analysis showed that oleuropein was the most abundant compound in olive leaf extracts (OLEs), its peak area recorded 39.15 %, 24.50% and 29.50%, respectively, followed by apigenin-7-glucoside for chloroform/methanol extract (peak area, 7.11%) and verbascoside for ethanol/water extract (peak area, 6.00%). The results exhibited that, there was a various degree of antifungal activity of the olive leaf extracts against six oral Candidal isolates (*Candida albicans, C. glabrata, C. krusei, C. guillermondii*, C. *parapsilosis* and *C. tropicalis*). Statistically; our data exhibit a highly significant difference between all concentrations of all olive leaf extracts (P-value < 0.05), except chloroform/methanol extract exhibit no significant between all the different concentrations on *Candida albicans* (P-value = 0.07). Nystatin was significantly related to all tested olive leaf extracts (P-value < 0.05); while Voriconazole showed no significant difference to all tested olive leaf extracts (P-value > 0.05). MIC for all plant extracts ranged from 35-40 µg/ml. The MIC for water extract recorded 35 µg/ml on *Candida albicans*, *C. glabrata, C. guillermondii*, *C. parapsilosis* and *C. tropicalis,* while on *C. krusei* recorded 40 µg/ml. On the other hand the MIC for chloroform/methanol extract recorded 35 µg/ml on all tested isolates except on *C. albicans* recorded 40 µg/ml.

[Mohamed A. Fareid. ***In Vitro:* Comparative study of Inhibitory Activity of *Olea europaea subsp. cuspidate* Leaf Extracts Against Oral Candidiasis.** *Researcher* 2015;7(11):11-20]. (ISSN: 1553-9865). http://www.sciencepub.net/researcher. 3

Keywords: Inhibitory activity, Olive leaf extracts, Candidiasis, HPLC.

1. Introduction

Candidiasis is an opportunistic infectious condition caused by a saprophytic fungus of the genus *Candida*, Oral candidiasis is predominately caused by *Candida albicans*, although other related *Candida* species may be involved. Candidiasis is a major cause of nosocomial infections, linked to a number of risk factors. Some species of the genus *Candida* are normally found in the flora of the human skin and mucosa (Eksi *et al*., 2013). They are still the most isolated yeast species regardless of body site and sampling time (Mnichowska-Polanowska *et al*., 2013). Olive tree (*Olea europaea*) is one of the most important fruit trees in Mediterranean countries. During the last fifteen years national production of olive oil has increased rapidly and there is an enormous potential for olive oil and table olive production in the KSA, especially in the Northern region (Al-Jouf, Gurayat, Hail and Tabuk) due to its temperate climate (Mabroka*, et. al*., 2014).

Olive leaves and fruits contain several constituents with pharmacological activities. It is known that the olive fruit, its oil and the leaves of the olive tree have a rich history of nutritional and medicinal uses (Soni *et al*., 2006). Oleuropeosits (oleuropein), flavones, flavonols and substituted phenols (tyrosol, hydroxytyrosol) are some phenolic compounds in the olive leaf extract (Benavente-Garcia *et al*., 2000). It has been reported by many researchers that the olive leaf extract has an antimicrobial activity because of its high phenolic content (Markin *et al*., 2003; Owen *et al*., 2003; Pereira *et al*., 2007; Sudjana *et al*., 2009; Aytul, 2010).

The most abundant compound in olive leaf extracts (OLEs) is oleuropein, followed by hydroxytyrosol (HT), and flavonoids (rutin and diosmin). OLE contains compounds with potent antimicrobial activities against bacteria, fungi, and mycoplasma (Huang *et al*., 2003; Dub and Dugani, 2013). Phenolic compounds isolated from olive leaf extract have been shown to inhibit the growth of *Eschesrichia coli*, *Klebslella pneumonia, Campylobacter jejuni, Helicobacter pylori,* methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli, Bacillus cereus, Salmonella typhi* and *Vibrio parahaemolyticu* (Aziz *et. al*., 1998; Yigit *et.al*., 2001; Owen *et. al*., 2003). Majgaine and Verma (2013) investigated the antifungal activity of extracts from *Olea cuspidata* and *Olea glandulifera* species. Olive leaf extract also inhibits many gram-negative and gram-positive bacteria, yeast, and parasites, including the malaria-causing *Plasmodium falciparum* (Markin, *et. al*., 2003).

Nystatin and fluconazole are effective agents in the treatment of various fungal infections (Ng and Wasan, 2003). They are the first drugs of choice for the treatment of certain candidiasis (Aguiar, *et al*., 2010). Unfortunately, the toxicities caused by polyene antifungals and resistance to fluconazole limit their use (Semis *et al*., 2012; Robinson, 2014). The search for novel antimicrobial agents derived from botanical sources never stopped, where plants provide a source of antimicrobial substances needed to manage some of the emerging resistant microbial species (Abreu and McBain, 2012).

In recent years, there was considerable public pressure to reduce the use of synthetic fungicides in medicine. Although, the use of synthetic fungicides in human fungal disease control has been successful in fungal disease treatment, several of these have been found to exhibit side-effects in the form of an allergic reaction. The alternative choice therefore would be the use of botanical fungicides, which are found to be largely non-phytotoxic, systematic and easily biodegradable in nature. Therefore, the current study aimed to evaluate the phenolic compounds of *Olea europaea subsp. cuspidate* leaves and achieve a comparative study to determine the anti-candidal effects of various olive leaf extracts prepared using different extraction procedures from olive collected from different districts of Ha'il province, Saudi Arabia.

2. Materials and methods

2.1. The Candidal isolates

Six candidal isolates (*Candida albicans, C. glabrata, C. krusei, C. guillermondii*, *C. parapsilosis* and *C. tropicalis*) were isolated from private dental clinics, in H'ail province, Saudi Arabia and identified by classical methods. Also, the identification of Candidal isolates was confirmed by using the API 20C *Candida* identification system (Bio-Merieux, Marcy I'Etoile, France).

2.2. Preparation of test microorganisms

All candida isolates were cultured twice at a 48 hr. interval before use on Potato dextrose agar (PDA, Difco, MI, Detroit, USA) slant at 35 °C for 24 hr. A loopful of PDA culture was inoculated in 50 ml of Malt extract broth (Difco, MI, Detroit, USA) and then incubated at 35 °C for 24 hr. (Arora and Kaur, 1999).

2.3. Preparation of olive leaf extracts

Olive leaves (*Olea europaea*) were collected during May, 2014 from different districts of Ha'il province, Saudi Arabia. The plant leaves were washed in running tap water to remove debris and dust particles and then rinsed in distilled water for 5 min, then removed and air dried under room temperature until constant weight. Olive leaves (30 g) were homogenized and extracted for 8 hr. in a Soxhlet apparatus with 150 ml of various solvents (Extract A: Chloroform/Methanol (50/50, v/v), (2% OLE, w/v); Extract B: Water (2% OLE, w/v); Extract C: Ethanol/Water (70/30, v/v), (2% OLE, w/v). The crude extracts were concentrated in a rotary evaporator and then transferred into sterile vials with 10 ml extraction solvent. Extracts were kept under refrigerated conditions until further use (Rauha *et al*., 2000, Sagdic *et al.,* 2002).

2.4. Phytochemical screening

a- Identification of phenolic acids

Phenolic compound were subsequently checked for purity by high pressure liquid chromatography (HPLC). HPLC grade water and MeOH were used for all analyses. Phosphoric acid buffer was made using HPLC grade $NH_4H_2PO_4$ and H_3PO_4. Phenolic extraction and hydrolysis Phenolic compounds in plant were extracted as described by Mattila *et al*., (2005). Approximately, 15 ml of 4N NaOH was added to 200 ml of each concentration of water extract in 50 ml Pyrex centrifuge tube purged with nitrogen and shaked for 2hr. in dark with a wrist-action shaker. After phenolic acids were liberated by alkaline hydrolysis, samples were acidified with ice - cold 6 N HC1 to reduce pH to 2. Samples were centrifuged at 3000 g and the supernatant was decanted into 250 ml separator/funnel. The supernatant was extracted with ethyl acetate (3:50 ml) with shaking for 10 Sec. and the mixture was allowed to settle for 5 min. between extractions. Ethyl acetate fractions were collected and pooled. The remaining pellet was diluted with 15 ml of distilled H_2O, vortex distributed and re-centrifuged at 3000 g. The second supernatant was re-extracted with ethyl acetate (3: 50 ml) as before and all ethyl acetate fractions were pooled. The phenolic acids-rich ethyl acetate fraction was dried by addition of anhydrous sodium sulfate and concentrated using a rotary vacuum evaporator at 35°C to dryness. The phenolic acids-rich residue was re-solubilized in 2.5 ml of MeOH and stored in a dark prior to separation and quantification by HPLC within 24 hr. of extraction.

b-HPLC analysis

Phenolic acids were separated by Shimaduz (Kyoto, Japan) HPLC apparatus (model, LC-4A) equipped with visible/ ultraviolet (UV) detector (model, SPD-2AS) at 280 nm and stainless steel column (25.0 cm X 4.6 mm i.d.) (Phenomenex Co., USA) coated with ODS, (RP-18). An aliquot of the sample suspended in MeOH was diluted with 10 mM phosphoric acid buffer (pH 3.5) to the same concentration as initial mobile phase (15% MeOH). Samples were next filtered through a 0.2 µm poly (tetrafluoroethylene) (PTFE) filter prior to injection. The two solvent systems consisted of MeOH (A) and 10 mM phosphoric acid buffer, pH 3.5 (B), operated at

following rate of 1.5 ml/min. The phosphoric acid buffer consisted of 10 mM $NH_4H_2PO_4$ adjusted to pH 3.5 with 10 m M H_3PO_4.

2.5. In vitro antifungal assay

The antimicrobial assay was performed using the agar diffusion method of Collins *et al.* (1995) with slight modifications. The test organisms were inoculated on Sabouraud dextrose agar (SDA) plates and spread uniformly using a sterile glass spreader. Wells of 5 mm diameter were made on the Sabouraud dextrose agar using a sterile cork borer. The cut agar disks were carefully removed by the use of sterilized forceps. To each well was introduced various concentrations (12.5, 25, 50, 75 and 100 µg/ml) of the extracts. Control experiments comprising inoculums without plant extract were set up. The plates were allowed to stand for one hour at room temperature (25 ±2°C) for diffusion of the substances to proceed before the growth of organisms commenced. The plates were incubated at 37°C for 24 hr. The zones of inhibition were then measured and recorded in mm diameter.

2.6. Determination of minimum inhibitory concentration (MIC) of olive leaf extracts (OLEs) on Candidal growth.

Olive leaf extracts that inhibited the growth of Candida isolates (*Candida albicans, C. glabrata, C. krusei, C. guillermondii*, *C. parapsilosis* and *C. tropicalis*) were investigated to determine the MICs using a broth-microdilution method. The Candida isolates were cultured overnight on mueller-hinton agar and then re-suspended in 1 ml mueller-hinton broth (OXOID CM 405) to obtain a final concentration of 100 CFU ml . Each extract was serially diluted with muellerhinton broth using methods approved by the National Committee for Clinical Laboratory Standards (M27-A) (NCCLS, 1997). After incubation, the MIC was determined as the lowest concentration of olive leaf extract for which there was no visible growth.

2.7. Statistical analysis

The statistical Package for Social Sciences (SPSS) version 15 was used in data analysis. All the parameters measured were expressed as means ± standard deviation. The difference between mean values was analyzed by Student's *t* test at the 5 % significance level. The confidence interval used for all statistical analyses was 95%. *P*-values less than 0.05 were significant.

3. Results

In this study, olive leaf extracts (OLEs) was tested for antifungal activity against six Candida isolates (*Candida albicans, C. glabrata, C. krusei, C. guillermondii*, *C. parapsilosis* and *C. tropicalis*) isolated from private dental clinics, in H'ail province, Saudi Arabia.

An HPLC chromatogram of phenolics in the chloroform/methanol extracted olive leaf extract (OLE) is shown in Figure 1 and the phenolic compounds distributions in different OLEs (chloroform/methanol, water and ethanol/water) are given in Table 1. Our results showed that oleuropein is the most abundant compound in OLEs, its peak area recorded 39.15%, 24.50% and 29.50%, respectively, followed by apigenin-7-glucoside for chloroform/methanol extract (peak area, 7.11%); hydroxytyrosol for water and ethanol/water extracts (peak area, 5.17% and 4.22%, respectively) and verbascoside for ethanol/water extract (peak area, 6.00%).

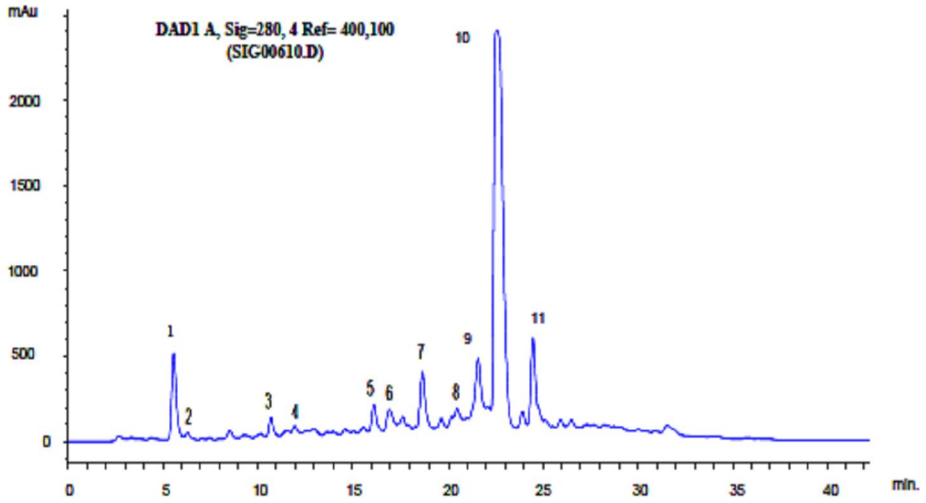

Fig. (1) Phenolic compounds of *Olea europaea L.* leave extracts.

Table (1): Peak number and peak area (%) of the main phenolic compounds present in olive leaf extract solutions

Peak No.	Phenolic compounds	Peak area %		
		Chloroform/methanol	Water	Ethanol/water
1	Hydroxytyrosol	4.22	5.17	2.25
2	Tyrosol	--	2.20	1.78
3	Catechin	1.33	2.11	2.20
4	Caffeic acid	0.95	1.42	1.03
5	Vanilic acid	2.50	2.31	3.05
6	Vanilin	2.43	2.15	2.55
7	Rutin	5.00	3.64	4.68
8	Luteolin-7-glucoside	2.55	2.41	1.95
9	Verbascoside	--	--	6.00
10	Oleurolin	39.15	24.50	29.50
11	Apigenin-7-glucoside	7.11	3.88	2.30

3.1. Susceptibility of *C. albicans* to different concentrations of olive leaf extracts.

Out of a total 3 olive leaf extracts (OLEs) (chloroform/methanol, water and ethanol/water) were tested for their *in vitro* inhibitory activities against *Candida albicans*. Our results showed that water and ethanol/water extracts were recorded higher inhibitory activity at 100 µg/ml concentration. The inhibition zone recorded 25.6 and 22.3 mm, respectively, while chloroform/ methanol recorded a lower inhibitory activity at the same concentration. The inhibition zone recorded 18.0 mm (Table 2). Statistically, our data exhibit a highly significant difference between all concentrations of water and ethanol/water extracts (P-value < 0.05), while chloroform/methanol extract exhibit no significant difference between all the different concentrations (P-value = 0.07).

Table (2): Susceptibility of *C. albicans* to different concentrations of olive leaf extracts.

Extract	Inhibition zone (mm) ± SD at different concentrations of olive leaf extracts						
	Control	12.5 µg/ml	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	P-value
Extract A	0.0	5.3 ±0.2	9.1 ±0.2	12.5 ±0.4	15.7 ±0.5	18.0 ±0.6	0.07
Extract B	0.0	4.1 ±0.8	10.6 ±0.4	15.0 ±0.6	20.5 ±0.3	25.6 ±0.4	0.00
Extract C	0.0	6.5 ±0.3	12.0 ±0.4	14.3 ±0.6	18.6 ±0.4	22.3 ±0.8	0.03

Extract A: Chloroform/Methanol (50/50, v/v), (2% OLE, w/v), Extract B: Water (2% OLE, w/v), Extract C: Ethanol/Water (70/30, v/v), (2% OLE, w/v).

3.2. Susceptibility of *C. glabrata* to different concentrations of olive leaf extracts.

Table (3) represents the inhibitory activity of tested olive leaf extracts (OLEs) (Chloroform/methanol, water and ethanol/water) against *C. glabrata*. Chloroform/methanol and water extracts were recorded higher inhibitory activity at 100 µg/ml concentration. The inhibition zone recorded 24.3 and 22.6 mm, respectively, while ethanol/water recorded a lower inhibitory activity at the same concentration, the inhibition zone recorded 18.5 mm. Statistically, our results showed that a highly significant difference between all concentrations of all olive leaf extracts (P-value < 0.05).

Table (3): Susceptibility of *C. glabrata* to different concentrations of olive leaf extracts.

Extract	Inhibition zone (mm) ± SD at different concentrations of olive leaf extracts						
	Control	12.5 µg/ml	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	P-value
Extract A	0.0	4.9 ±0.2	11.6 ±0.5	16.5 ±0.3	20.1 ± 0.5	24.3 ±0.7	0.00
Extract B	0.0	5.2 ±0.7	10.5 ±0.3	13.6 ±0.2	16.5 ±0.3	22.6 ±0.2	0.01
Extract C	0.0	4.4 ± 0.5	8.6 ±0.2	11.5 ±0.3	15.6 ±0.2	18.5 ±0.3	0.03

Extract A: Chloroform/Methanol (50/50, v/v), (2% OLE, w/v), Extract B: Water (2% OLE, w/v), Extract C: Ethanol/Water (70/30, v/v), (2% OLE, w/v).

3.3. Susceptibility of *C. krusei* to different concentrations of olive leaf extracts.

Table (4) represents the susceptibility of *C. krusei* to different concentrations of olive leaf extracts. Our results showed that all tested olive leaf extracts (OLEs) (Chloroform/methanol, water and ethanol/water) exhibit antifungal effects against *Candida krusei*. Chloroform/methanol and water extracts were recorded higher inhibitory activity at 100 µg/ml concentration. The inhibition zone recorded 25.0 and 20.9 mm, respectively, while ethanol/water extract recorded a lower inhibitory activity at the same concentration, its inhibition zone recorded 17.5 mm. Statistically, our data showed that there is a highly significant difference between all concentrations of all olive leaf extracts (P-value < 0.05).

Table (4): Susceptibility of *C. krusei* to different concentrations of olive leaf extracts.

Extract	Inhibition zone (mm) ± SD at different concentrations of olive leaf extracts						
	Control	12.5 µg/ml	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	P-value
Extract A	0.0	5.8 ±0.2	11.0 ±0.3	16.5 ±0.6	20.8 ±0.5	25.0 ±0.3	0.00
Extract B	0.0	4.7 ±0.3	9.5 ±0.3	11.1 ±0.2	16.1 ±0.5	20.9 ±0.6	0.01
Extract C	0.0	0.00	4.6 ±0.2	9.4 ±0.2	13.1 ±0.5	17.5 ±0.3	0.00

Extract A: Chloroform/Methanol (50/50, v/v), (2% OLE, w/v), Extract B: Water (2% OLE, w/v), Extract C: Ethanol/Water (70/30, v/v), (2% OLE, w/v).

3.4. Susceptibility of *C. guillermondii* to different concentrations of olive leaf extracts.

Table (5) represents the susceptibility of *C. guillermondii* to different concentrations of olive leaf extracts. Our results showed that a highly inhibitory activity to Chloroform/methanol followed by water extract against *C. guillermondii* at 100 µg/ml concentration. The inhibition zone recorded 22.9 and 21.5 mm, respectively, while ethanol/water extract recorded a lower inhibition zone (19.3 mm) at the same concentration. Statistically; our data exhibit a highly significant difference between all concentrations of all olive leaf extracts (P-value < 0.05).

Table (5): Susceptibility of *C. guillermondii* to different concentrations of olive leaf extracts.

Extract	Inhibition zone (mm) ± SD at different concentrations of olive leaf extracts						
	Control	12.5 µg/ml	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	P-value
Extract A	0.0	4.5 ±0.3	9.8 ±0.2	13.9 ±0.5	18.5 ±0.3	22.9 ±0.3	0.00
Extract B	0.0	4.0 ±0.5	8.5 ±0.5	11.8 ±0.2	16.0 ±0.6	21.5 ±0.3	0.00
Extract C	0.0	3.8 ±0.2	7.7 ±0.2	10.5 ±0.3	16.0 ±0.6	19.3 ±0.7	0.00

Extract A: Chloroform/Methanol (50/50, v/v), (2% OLE, w/v), Extract B: Water (2% OLE, w/v), Extract C: Ethanol/Water (70/30, v/v), (2% OLE, w/v).

3.5. Susceptibility of *C. parapsilosis* to different concentrations of olive leaf extracts.

Table (6) represents the susceptibility of *C. parapsilosis* to different concentrations of olive leaf extracts. Our data exhibit higher inhibitory activity to chloroform/methanol and water extracts against *C. parapsilosis* at concentration 100 µg/ml. The inhibition zone recorded 26 and 24.3 mm, respectively, while ethanol/ water extract recorded a lower inhibitory activity at the same concentration It inhibition zone recorded 19.9 mm. Statistically, our data exhibit a highly significant difference between all concentrations of all olive leaf extracts (P-value < 0.00).

Table (6): Susceptibility of *C. parapsilosis* to different concentrations of olive leaf extracts.

Extract	Inhibition zone (mm) ± SD at different concentrations of olive leaf extracts						
	Control	12.5 µg/ml	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	P-value
Extract A	0.0	5.6 ±0.3	11.0 ±0.1	15.1 ±0.3	22.5 ±0.5	26.0 ±0.6	0.00
Extract B	0.0	5.8 ±0.8	10.5 ±0.3	14.5 ±0.3	19.3 ±0.6	24.3 ±0.4	0.00
Extract C	0.0	4.9 ±0.6	9.3 ±0.3	11.7 ±0.6	16.5 ±0.2	19.9 ±0.3	0.02

Extract A: Chloroform/Methanol (50/50, v/v), (2% OLE, w/v), Extract B: Water (2% OLE, w/v), Extract C: Ethanol/Water (70/30, v/v), (2% OLE, w/v).

3.6. Susceptibility of *C. tropicalis* to different concentrations of olive leaf extracts.

Table (7) represents the susceptibility of *C. tropicalis* to different concentrations of olive leaf extracts. Our data showed that the inhibitory activity of tested olive leaf extracts (Chloroform/methanol, water and ethanol/water) against *C. tropicalis*. Our results exhibit higher inhibitory activity to all olive leaf extracts at concentration 100 µg/ml. The inhibition zone recorded 21.5, 24.8 and 22.1 mm, respectively. Statistically, our data exhibit a highly significant difference between all concentrations of all olive leaf extracts (P-value = 0.00).

Table (7): Susceptibility of *C. tropicalis* to different concentrations of olive leaf extracts.

Extract	Inhibition zone (mm) ± SD at different concentrations of olive leaf extracts						
	Control	12.5 µg/ml	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml	P-value
Extract A	0.0	3.9 ±0.4	7.1 ±0.5	11.0 ±0.2	16.3 ±0.4	21.5 ±0.3	0.00
Extract B	0.0	5.8 ±0.3	10.0 ±0.6	16.0 ±0.7	21.0 ±0.6	24.8 ±0.8	0.00
Extract C	0.0	5.2 ±0.6	9.1 ±0.2	13.1 ±0.1	17.5 ±0.5	22.1 ±0.3	0.00

Extract A: Chloroform/Methanol (50/50, v/v), (2% OLE, w/v), Extract B: Water (2% OLE, w/v), Extract C: Ethanol/Water (70/30, v/v), (2% OLE, w/v).

3.7. The inhibition zone diameters of the tested isolates against different antifungal agents.

Table (8) represents the inhibition zone diameters shown by the antifungal agents on the Candidal isolates, there were varying degree of inhibition of all the isolates used in the study. *C. albicans* and *C. tropicalis* isolates exhibited resistance towards Clotrimazole and Voriconazole, respectively. While, *C. krusei* and *C. parapsilosis* were sensitive to all tested antifungal agents. None of the Candidal isolates was shown resistance towards Nystatin and Amphotericin B.

Table (8): The inhibition zone diameters of the tested isolates against different antifungal agents.

Candidal isolates	Inhibition zone of tested Candida isolates (mm)				
	Nystatin	Fluconazole	Amphotericin B	Clotrimazole	Voriconazole
C. albicans	18.0±0.4 S	16.6±0.8 S	20.3±0.4 S	14.0 ±1.3 R	26.0 ±0.6 S
C. glabrata	16.6±0.8 S	14.0±0.6 R	22.0±0.6 S	15.6 ±1.7 S	23.3 ±1.7 S
C. krusei	17.0±0.6 S	15.0±0.6 S	17.6±0.4 S	22.6 ±0.4 S	14.0 ±0.6 S
C. guillermondii	15.0±0.6 S	12.5±0.3 R	22.3±0.4 S	17.6 ±1.5 S	23.0 ±1.3 S
C. parapsilosis	15.6±0.3 S	24.0±0.3 S	19.0±0.6 S	21.0 ±1.3 S	29.3 ±1.1 S
C. tropicalis	19.0±0.3 S	15.6±0.4 S	20.8±0.5 S	18.0 ±0.6 S	13.0 ±1.3 R

R, Resistance; S, Sensitive.

Table (9): Paired Samples t-Test of anti-fungal agents and Olive leaf extracts.

Paired Samples Test					
Pair No.	Antifungal agents	Extracts	t	Sig. (2-tailed)	Decision (α = 0.05)
Pair 1	Nystatin	Chloroform/methanol	3.771	0.013	Significance
Pair 2	Nystatin	Water	9.562	0.000	Significance
Pair 3	Nystatin	Ethanol/water	4.748	0.005	Significance
Pair 4	Fluconazole	Chloroform/methanol	3.885	0.012	Significance
Pair 5	Fluconazole	Water	4.889	0.005	Significance
Pair 6	Fluconazole	Ethanol/water	2.177	0.081	Non-significance
Pair 7	Amphotericin B	Chloroform/methanol	1.665	0.157	Non-significance
Pair 8	Amphotericin B	Water	2.863	0.035	Significance
Pair 9	Amphotericin B	Ethanol/water	0.423	0.690	Non-significance
Pair 10	Clotrimazole	Chloroform/methanol	5.432	0.003	Significance
Pair 11	Clotrimazole	Water	2.826	0.037	Significance
Pair 12	Clotrimazole	Ethanol/water	0.963	0.380	Non-significance
Pair 13	Voriconazole	Chloroform/methanol	0.520	0.625	Non-significance
Pair 14	Voriconazole	Water	0.727	0.500	Non-significance
Pair 15	Voriconazole	Ethanol/water	0.553	0.604	Non-significance

Table (10): Minimum inhibitory concentration (µg/ml) of olive leaf extracts on Candidal isolates growth.

Candidal isolates	Olive leaves extract concentrations (µg/ml)											
	Chloroform/Methanol				Water				Ethanol/Water			
	40	35	30	25	40	35	30	25	40	35	30	25
C. albicans	-ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve
C. glabrata	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve
C. krusei	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
C. guillermondii	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve
C. parapsilosis	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	+ve	+ve	+ve
C. tropicalis	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve

(+ve), growth observed; (-ve), no growth observed.

3.9. Paired Samples t-Test of anti-fungal agents and olive leaf extracts.

To test the hypothesis of no difference or no relationship between the size of inhibition zone of antifungal drugs and all tested olive leaf extracts paired t-test was performed (Table 9). For seven pairs, 46.6 % of pairs rejected null hypothesis in favor of alternate hypothesis, there is no significant difference between the mean values of Amphotericin B, chloroform/methanol and ethanol/water inhibition zone (P-value > 0.05). Nystatin was significantly related to all tested olive leaf extracts (P-value < 0.05); while Voriconazole showed no significant difference to all tested olive leaf extracts (P-value > 0.05).

3.10. Minimum inhibitory concentration (µg/ml) of olive leaf extracts on Candida isolates growth.

MICs of the olive leaf extracts (chloroform/methanol, water, and ethanol/water) were calculated by using a broth-microdilution method (Table 10). Our results showed that the MIC for all OLEs ranged from 35-40 µg/ml. The MIC for water extract recorded 35 µg/ml on *Candida albicans*, *C. glabrata, C. guillermondii*, *C. parapsilosis* and *C. tropicalis,* while on *C. krusei* recorded 40 µg/ml. Also, the MIC for ethanol/water extract recorded 40 µg/ml on all tested isolates, while on *C. tropicalis* recorded 35 µg/ml. On the other hand the MIC for chloroform/methanol extract recorded 35 µg/ml on all tested isolates except on *C. albicans* recorded 40 µg/ml.

4. Discussion

The products of olive tree that can live for centuries are known for many years with their beneficial effects on health (Soler-Rivas *et al*., 2000). Its reported by some researchers that the oleuropein which is included in these products has a lot of pharmacological properties including antioxidant, antimicrobial, antiinflammatory, anticarcinogenic and antiviral activities (Owen *et al.,* 2003; Visioli *et al.,* 2002; Micol *et al*., 2005; Sanchez *et al*., 2007).

The phytochemical screening of olive leaf extracts (*Olea europaea subsp. cuspidate*) showed that, the HPLC analysis successfully provided the presence of 11 phenolic compounds. The major phenolic compounds isolated from leaves of *Olea europaea* were Hydroxytyrosol, Tyrosol, Catechin, Caffeic acid, Vanilic acid, Vanilin, Rutin, Luteolin-7-glucoside, Verbascoside, Oleurolin and Apigenin-7-glucoside. Oleuropein recorded the highest concentrations in OLEs (its peak area, 39.15%, 24.50% and 29.50%, respectively) followed by apigenin-7-glucoside for chloroform/methanol extract (Peak area, 7.11%) and verbascoside for ethanol/water extract (Peak area, 6.00%). Our finding was in accordance with Korukluoglu, *et al.,* (2010), Erdohan and Turhan (2011) and Khattab, *et al*., (2015). Altiok *et al*., (2008) examined the effect of different solvents on the total phenolic content of OLEs and oleuropein abundance in extracts. They obtained an extract with highest total phenolics (10.3 µ total phenolics/g leaf) and oleuropein (92 µg oleuropein/g leaf) content using 70% ethanol. Pereira *et al.*, (2007) used water as solvent to obtain OLE and they detected 26.5 µg oleuropein/g lyophilized olive leaves extract. Similarly, our data showed variation between the amounts of phenolic compounds depend upon the type of solvent used in the extraction process, the peak area of oleuropein recorded 39.15 % in chloroform/methanol extract while in ethanol/water recoded 29.50 %.

Markin *et al.,* (2003) reported that aqueous extract of olive leaf (15%) killed *C. albicans* within 24 hr. Pereira *et al.* (2007), reported the olive leafs antimicrobial capacity in the following order *Bacillus cereus ~ Candida albicans > E. coli > S. aureus > P. aeruginosa* and there is no selectivity between gram positive and gram-negative bacteria. They also reported that the antimicrobial mechanism of olive leaf extract is denaturating the proteins and effecting

cell membrane permeability. Also, Lee and Lee (2010) reported that the combined phenolics mixture which was prepared from olive leaf extract showed inhibition effects against *B. cereus* and *S. enteritidis*. Owen *et al.* (2003) also reported that olive leaf extracts presented antimicrobial activity against *E. coli, S. aureus, B. cereus, S. typhi* and *V. parahaemolyticus*.

The antifungal activities of olive leaf extracts (chloroform/methanol, water, and ethanol/water) against tested yeasts were quantitatively assessed by the presence or absence of MIC (µg/ml) and inhibition zone diameters (mm). MIC for all plant extracts ranged from 35-40 µg/ml. The MIC for water extract recorded 35 µg/ml on *Candida albicans*, *C. glabrata, C. guillermondii*, *C. parapsilosis* and *C. tropicalis,* while on *C. krusei* recorded 40 µg/ml. Also, the MIC for ethanol/water extract recorded 40 µg/ml on all tested candidal isolates, while on *C. tropicalis* recorded 35 µg/ml. All tested Candidal isolates showed various degree of antifungal sensitivity to chloroform/methanol, water, and ethanol/water extracts of olive leaves. Chloroform/methanol and water extracts were recorded higher inhibitory activity at 100 µg/ml concentrations on most candidal isolates. Statistically; our data exhibit a highly significant difference between all concentrations of all olive leaf extracts (P-value < 0.05), The results in our study, has similarity between the other studies (Markin *et al*., 2003; Owen *et al*., 2003; Pereira *et al*., 2007; Sudjana *et al*., 2009; Lee and Lee, 2010; Gökmen *et al*., 2014) about the anti-candidal activity of olive leaf extracts.

The hypothesis of no difference or no relationship between the size of inhibition zone of antifungal drugs and all tested olive leaf extracts paired t-test was performed. For seven pairs, 46.6 % of pairs rejected null hypothesis in favor of alternate hypothesis, there is no significant difference between the mean values of Amphotericin B, Chloroform/methanol and Ethanol/water inhibition zone (P-value > 0.05). Nystatin was significantly related to all tested ethanolic plant extracts (P-value < 0.05); while Voriconazole showed no significant difference to all tested olive leaf extracts (P-value > 0.05). Our results showed that the MIC for all plant extracts ranged from 35-40 µg/ml. The MIC for water extract recorded 35 µg/ml on *Candida albicans*, *C. glabrata, C. guillermondii*, *C. parapsilosis* and *C. tropicalis,* while on *C. krusei* recorded 40 µg/ml. this finding was in accordance with Halawi, *et al*., (2015), they showed that antifungal activity against the growth of all Candidal isolates under investigation, with the lowest recorded MIC and MFC of 2.5 and 15 mg/ml, respectively for both extracts.

In conclusion, the present study showed that Oleuropein recorded the highest concentrations in OLEs, its peak area recorded 39.15%, 24.50% and 29.50%, respectively) followed by apigenin-7-glucoside for chloroform/methanol extract (peak area, 7.11%) and verbascoside for ethanol/water extract (peak area, 6.00%). Nystatin was significantly related to all tested olive leaf extracts (P-value < 0.05). MIC for all olive leaf extracts ranged from 35-40 µg/ml. The MIC for water extract recorded 35µg/ml on *Candida albicans*, *C. glabrata, C. guillermondii*, *C. parapsilosis* and *C. tropicalis,* while on *C. krusei* recorded 40 µg/ml.

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11/2/2015