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THE EFFECT OF ESSENTIAL OILS OF SELECTED PLANTS ON CLINICAL ISOLATES OF *CANDIDA* SPECIES GROWTH, TRANSITION AND BIOFILM FORMATION

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Candida species are one of common opportunistic pathogens in humans. Detection of antifungal resistance between candida species become increasing, a great interest in creating new antifungals utilizing natural sources like plant essential oils (EOs) . ***This study aimed*** *to evaluate the effect of EOs of selected plants on Candida species growth, transition, and biofilm formation as well on the expression levels of certain virulence genes.* ***Patients and methods:*** *Fifty candida isolates were collected from napkin dermatitis and oral candidiasis patients, different Candida species were identified phenotypically. Essential oils for some medicinal plants (basil, clove, garlic, and peppermint) were extracted by hydro-distillation. Their antifungal activity was assessed by minimal inhibitory concentration methods. In addition the effect of EOs on different Candida ultrastructure was detected by scanning electron microscope, the antibiofilm characteristics were examined by microtiter plate method. The ability of tested EOs to interfere with transition of candida from blastopore to hyphae form was evaluated. The effect of basil and clove oils on various virulent genes was determined by analyzing the expression levels of HWP, ALS3, SAP3 genes by RT-PCR.* ***Results****: C. albicans was the most prevalent species. For all tested Candida isolates, the EOs displayed a high antifungal activity. The EOs decreased the Candida transition, disrupted the Candida ultrastructure and suppressed their ability of biofilm formation, clove oil exposure significantly downregulated the expression of HWP1, ALS3 and SAP3 genes, in addition basil oil downregulated the expression of ALS3 and SAP3.****Conclusion****: This study concluded that clove oil is the best essential oil among other EOs that inhibited growth, transition and biofilm formation by lowest MIC and downregulated HWP1, AlS3 and SAP3 genes, which suggest their potential application for its use as antifungal therapy.*

Keywords: *Essential oil; C. albicans; Biofilm; HWP1; ALS3; SAP3 genes*

INTRODUCTION

Fungi have been found as one of the most frequent causes of human disease since the late 20th century, and immunocompromised patients continue to be most susceptible[1&2]. One of the commensals in the human body, *Candida spp.* is well known to cause opportunistic superficial and invasive infections[3]. *Candida* species transform from commensals to pathogens due to their virulent characteristics, such as their ability to adhere to host tissue, invasive biomedical devices (catheter), produce

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biofilms, and release extracellular hydrolytic enzymes.[4].

Candida albicans is most frequently isolated from HAI (Hospital Acquired Infections). Other non- *Candida albicans* species showed a growing incidence against nosocomial blood stream infections[5] . Systemic candidiasis is required several genes including *ALS1*, *ALS3*, and *HWP1*, to be expressed.[6] .

Enzymes like secreted aspartyl proteinases (*SAPs*) play a role in the invasion of *C. albicans* to degrade host proteins and invade tissues and organs[7] . The activation of other genes, such as *HWP1*, can support the effects of *SAPs* on *C. albicans* pathogenicity. The hyphal cell wall protein that is encoded by the hyphal-specific adhesion gene *HWP1* promotes *C. albicans* adherence to different surfaces[8] .

The anti-Candida medications that are currently available include azole derivatives like fluconazole and echinocandins like caspofungin that inhibit the formation of ergosterol [9]and cell wall[10]. Unfortunately, resistance to these conventional treatments is progressively rising.[11-14] . Due to the existence of phytochemicals that can result in the development of new drugs, medicinal plants are currently regarded as a possible source for new therapeutic agents.[15] . This study aimed to evaluate the effect of EOs of selected plants on *Candida species* growth, transition, and biofilm formation as well on the expression levels of certain virulence genes.

MATERIALS AND METHODS

Ethical statement

This work has been approved by the Local Ethical Committee of the Faculty of Medicine, Assiut University (IRB: 17101926).

Plant material and preparation of essential oils

Fresh leaves of *Ocimum basilicum* (Basil) and and *Mentha piperita* (Peppermint) (family Lamiaceae) and, collected from Medicinal plants station, Faculty of Pharmacy, Assiut university [voucher specimens (Aun-phg-0002031) and (Aun-phg-0002007) respectively were kept in Pharmacognosy Department Museum], flower buds of *Syzygium aromaticum* (Clove) (family Myrtaceae) and bulbs of *Allium sativum* (Garlic) (family Liliaceae) were purchased from Egyptian Markets. Extraction of volatile oil was by hydro-distillation of the plants by using Clevenger type apparatus[16] .

Identification of the main constituents of essential oils by Gas Chromatography Mass Spectrometry (GCMS)

The analysis of the oil samples was performed using GC-MS by injection onto a DB-5 column (7890A Agilent; 30 m×0.25 mm I. D., 0.25μm film thickness) on a 6890 N gas chromatograph (Agilent) coupled to a 5975B mass spectrometer (Agilent) as described previously [17] .

Identification of *Candida* isolates

This study was conducted on 50 candida isolates, 25 candida isolates were isolated from Hematological malignancy patients admitted to Clinical Hematology Unit, Internal Medicine Department, Assiut University Hospital. In addition, 25 Candida isolates were isolated from napkin dermatitis patients attended the outpatient clinics of pediatrics at Al-Azhar Assiut University Hospital, all isolates were transported aseptically to the Microbiology Laboratory of Assiut University; all isolates were streaked on the Sabouraud's dextrose agar (SDA) (Himedia Company, India). The isolated yeasts have been identified phenotypically by Germ tube formation[18], Hicrome Candida Differential agar (Himedia, Mumbai, India) [19]and Cornmeal agar (Himedia, Mumbai, India)[20]

Antifungal susceptibility

Determination of MIC of Clotrimazole drug and Essential oils

The minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of essential oils and clotrimazole were determined by the broth micro dilution method.[21].

Disc diffusion method

Itraconazole (10 g), Voriconazole (1 g), Clotrimazole (10 g), Nystatin (100 U), and Amphotericin-B (100 U) were the five antifungal drugs tested in an antifungal susceptibility test on *Candida* isolates by disc diffusion method (Himedia, India). Briefly, each isolate had an inoculum with a turbidity of 0.5 McFarland level in sterile 0.85% saline, which was streaked on Mueller-Hinton agar and supplemented with 0.5 g/ml methylene blue dye and 2% glucose. Plates were then

incubated at 35°C for 24 hours. According to CLSI interpretative breakpoints, inhibition zones were interpreted[22].

Effect of the essential oils on *C. albicans* transition from blastospore to hyphal Form

In 3 mL of Sabouraud dextrose broth that was supplemented with 0.1% glucose and 10% foetal bovine serum (FBS), *C. albicans* (10^5) were suspended, the sub-MIC of EO was added directly to the culture medium. The cultures were incubated at 37 °C to promote the formation of hyphae. The cultures were examined under a microscope and photographed after 6 hours of incubation to describe the morphology of *C. albicans*[23] .

Effect of clotrimazole and essential oils on *Candida albicans* ultrastructure

In this step, we used sub-MIC of EO. The Candida isolates (10^7cells) were cultured in Sabouraud dextrose broth in the presence of sub-MIC of EO. The *Candida* isolates that were cultivated without the addition of EO served as the negative control, while the *Candida* isolates that were cultured with clotrimazole sub-MIC served as the positive control (clotrimazole was used as a standard as all tested candida isolates were sensitive to it by both disc diffusion and MIC methods). The cells were cultured for 24 hours before being fixed in 3% (v/v) glutaraldehyde in PBS and dehydrated in ethanol at increasing concentrations (10%, v/v, increments to 100%) and were examined by scanning electron microscope[23] .

Antibiofilm effect of essential oils on different *Candida* species

The testing of the ability biofilm formation was performed using a 96-well micro titer plate Briefly, overnight cultures of *Candida* isolates were diluted 1:100 into 15 mL of Sabouraud broth with a final concentration of 8% glucose (Sigma-Aldrich, USA) in presence of series of essential oils and clotrimazole solutions ranging from 100 to 0.2mg/ml with two fold dilution (as that were used in MIC test). For 24 hours, cultures were incubated at 37 °C. The generated biofilm was then air dried after being incubated and rinsed three times with 200 µL of distilled water. After that, each well received 100 µL of 0.2% crystal violet (Merck KGaA, Germany), and the plate was incubated for 20 minutes to allow for biofilm staining. Wells were treated with 200 µL of 95% ethanol (SigmaAldrich, USA) to dissolve the crystal violet crystals after being rinsed three times with distilled water and dried by air. A microplate reader was used to measure the crystal violet's intensity at 570 nm after the plate had been incubated for 30 minutes (Epoch, USA). By comparing the absorbance values of the essential oil-treated wells with the untreated control wells, the ability of essential oils to affect biofilm formation was measured.[24] .

Effect of essential oils on *C. albicans HWP1, SAP3 and ALS3* gene expression

Different *Candida albicans* isolates (5×10^6 cells) were cultured in Sabouraud dextrose broth in the presence and absence of sub-MIC of EO at 30℃ for 6 h. The study also included a positive control (culture of different *Candida* albicans isolates in the presence of MIC ml of clotrimazole)[23]

RNA extraction

According to the manufacturer's instructions, total RNA was extracted from C. albicans isolates using the ABT total RNA mini extraction kit (Applied Biotechnology, Ankara, Turkey). A NanoDrop spectrophotometer (NanoDrop 2000C, ThermoFisher, USA) was used to measure the RNA concentration and purity**.**

Reverse transcription

500 ng of RNA was reverse transcribed using the ABT H-minus cDNA synthesis kit (Applied Biotechnology, Ankara, Turkey) in accordance with the instructions that were provided by the manufacturer.

Real time-qPCR analysis

Quantitative real-time PCR was applied to measure the expression levels of the housekeeping gene ACT1 and the virulence genes HWP1, SAP3, and ALS3 in *Candida albicans*. Primers used were listed in the **table (1)**. The Step One Plus Real-Time PCR Systems (Applied Biosystems, USA) were used to measure the expression levels of genes in a 20µl PCR reaction mixture including 10 µl of iQ SYBR Green Master Mix (Applied Biotechnology, Ankara, Turkey), 0.5 µl of each primer solution (10 µM), 5 µl cDNA sample and water was added to complete the volume to 20 µl. Amplification conditions included 95 ◦C for 5 min, 40 cycles of 95 ◦C for 15 sec,

annealing for 25 sec according to temperatures reported in **(Table 1)**, and 72 ◦C for 45 sec.

The results were analyzed using the 2−ΔΔCt (Livak) relative expression method[23] .

Statistical Analysis

The mean and standard deviation values were calculated for each group in each test. Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests and showed non-parametric (not normal) distribution. **Kruskal Wallis** test was used to compare between more than two groups in non-related samples **Mann Whitney** was used to compare between two groups in non-related samples. **Pearson's** correlations were used to analyze the correlation between different parameters within the combined groups.The significance level was set at $P < 0.05$. Statistical analysis was performed with IBM® SPSS® Statistics Version 26 for Windows.

RESULTS AND DISCUSSION

Results

Preparation and characterization of essential oils tested in the study

Extraction of essential oils by hydro-distillation of the plants

The yield of essential oils from (basil, clove, garlic and peppermint) were represented in **(Table 2)**.

Table 1: Primer sequences used for the qRT-PCR.

Gene	Primer Sequence (50 à 30)	Annealing temp	Reference
ACT1	Forward: GACAATTTCTCTTTCAGCACTAGA	56°C	23
	Reverse: GCTGGTAGAGACTTGACCAACCA		
HWP1	Forward: GCTCAACTTATTGCTATCGCTTATTA	56°C	23
	Reverse: GACCGTCTACCTGTGGGACAGT		
SAP3	Forward: GGACCAGTAACATTTTTATGAGTTTTGAT	53°C	23
	Reverse: TGCTACTCCAACAACTTTCAACAAT		
ALS3	Forward: CCAGTGTTCCAACAACTGAA	59°C	25
	Reverse; GAACCGGTTGTTGCTAT		

Table 2: Extraction yields of essential oils of investigated plants.

Plant name	Weight (kg)	Volume (ml)	Yield (v/w%)
Ocimum basilicum	2.1	7	0.33
Syzgium aromatic	3	15	0.5
Allium sativum	9.5	11.9	0.13
Mentha piperita	4.53	15.1	0.33

Identification of the main constituents of essential oils by Gas Chromatography Mass Spectrometry (GCMS)

In this study, the main constituents of basil oil were bornyl acetate (6.74%), α-Farnesene (4.10%), Cubenol (3.48%), Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)- (3.26%), and α-Cubebene (2.93%) **(Table 3)**, the main constituents of clove oil were eugenol (16.94%), α-elemen (11.91%), Isoeugenol (11.36%), caryophyllene (7.85%), and Cadina-1(10),4-diene (5.49%) **(Table 4)**, the main constituents of garlic oil were dimethyl trisulfide (2.43%), tetrasulfide, di-2-propenyl (1.61%), 1,2-dithiol-1-ium, 3,4,5-trimethyl-, bromide (1.28%), Dodecane (1.25%), and Trisulfide, di-2-propenyl (1.25%) **(Table 5)** and the main constituents of peppermint oil were humulene (7.21%), p-Mentha-6,8-dien-2-one (carvone) (5.47%), caryophyllene (5.09%), p-Menth-8-en-2-ol (4.62%), and α-phellandrene (3.83 %) **(Table 6)**.

Table 3: Chemical composition of basil essential oil by Gass Chromatography Mass Spectrophotometry.

RT	Compound Name	Area %
8.23	1R-α-Pinene	1.72
8.65	Camphene	0.74
8.88	1-Octen-3-ol	2.10
9.07	α-Phellandrene	1.35
9.26	α-Pinene	2.10
9.33	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)	1.21
10.36	Eucalyptol	2.93
10.61	Trifluoroacetyl-α-terpineol	1.11
11.70	1,6-Octadien-3-ol, 3,7-dimethyl- (linalool)	2.34
14.39	Borneol	1.82
15.08	Estragole	2.66
15.39	Benzene, 1-methoxy-4-(1-propenyl)-	0.89
16.08	Fenchyl acetate	1.37
19.01	Bornyl acetate	6.74
21.64	Eugenol	2.68
22.46	Isoeugenol	2.36
24.88	α –Farnesene	4.10
25.19	Isocaryophillene	0.95
25.95	α-Caryophyllene	1.71
26.15	Isoledene	2.54
26.72	α-Cubebene	2.93
27.17	γ-Elemene	2.64
27.27	Guaia-1(10),11-diene	2.17
27.51	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4 -methylene-1-(1-methylethyl)-	3.26
31.05	Cubenol	3.48
32.63	α-Cadinol	0.78

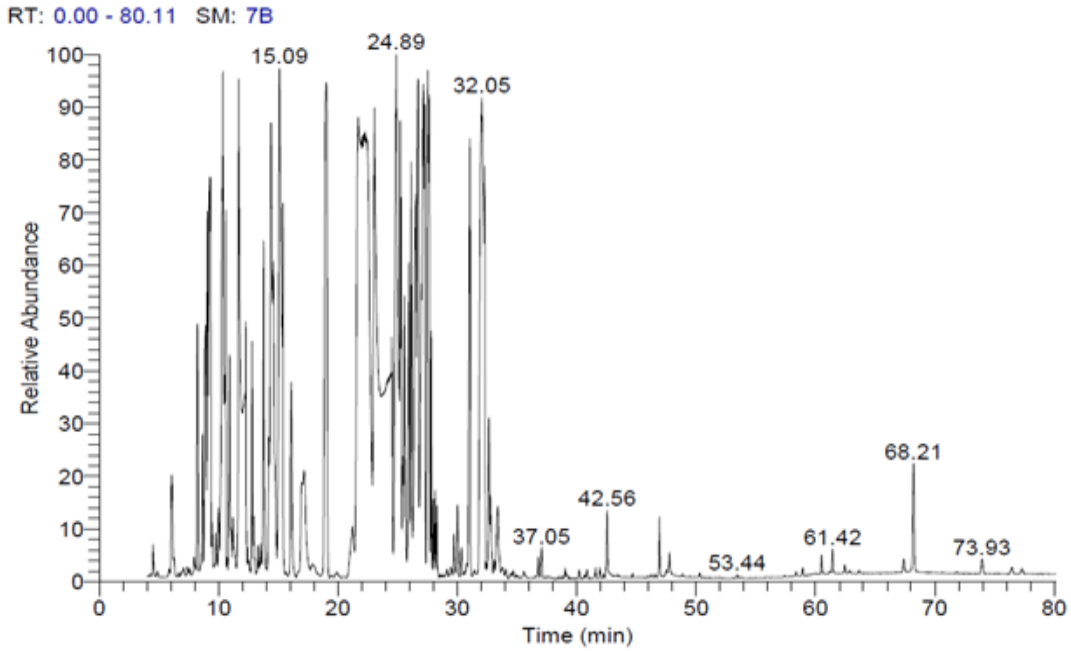

Fig. 1: GC chromatogram of basil oil**.**

Table 4: Chemical composition of clove essential oil by Gass Chromatography Mass Spectrophotometry.

RT	Compound Name	Area%
9.05	α-Pinene	0.53
9.26	Bicyclo[3.1.1]heptane,6,6-dimethyl-2-methylene-,(1S)-	0.72
10.40	Eucalyptol	1.78
11.42	2-Nonanone	0.96
11.57	2-Nonanol	0.61
15.12	Methyl salicylate	1.79
21.61	Isoeugenol	11.36
23.35	Copaene	0.79
23.40	Ylangene	2.20
24.68	Caryophyllene	7.85
25.47	Isocaryophillene	3.55
25.74	α-cubebene	1.15
25.96	α-Elemene	11.91
26.55	α-Farnesene	3.19
27.31	Eugenol	16.94
27.61	Cadina-1(10),4-diene	5.49
30.07	Calarene epoxide	1.59

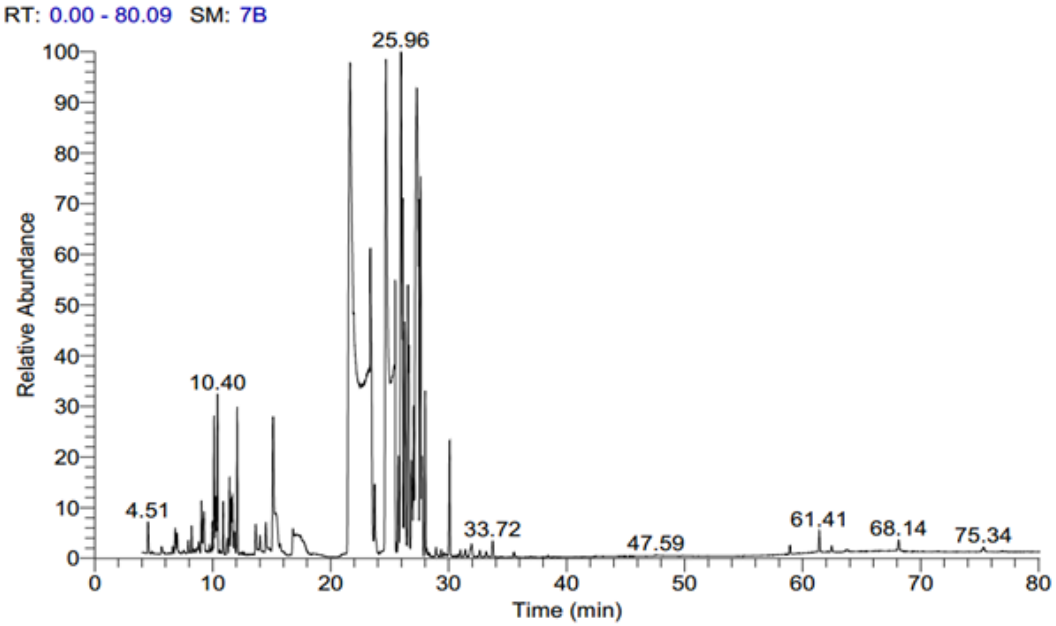

Fig. 2: GC chromatogram of clove oil**.**

Table 5: Chemical composition of garlic essential oil by Gass Chromatography Mass Spectrophotometry.

RT	Compound Name	Area %
4.49	Toluene	0.51
6.35	Allyl sulfide	0.52
8.30	Disulfide, methyl 1-propenyl	0.56
8.66	2-Furancarboxaldehyde, 5-methyl	0.51
9.28	Dimethyl trisulfide	2.43
11.19	Naphthalene, decahydro-, cis	0.65
11.49	Diallyl disulphide	1.17
12.11	Tetrasulfide, di-2-propenyl	1.61
12.20	2-Vinyl-1,3-dithiane	1.22
13.48	Trisulfide, methyl 2-propenyl	13.48
14.46	1,3,5-Trithiane	0.95
14.63	Dodecane	1.25
14.69	Tetradecane	0.68
15.42	3-Vinyl-1,2-dithiacyclohex-4-ene	1.20
16.35	1,2-Dithiol-1-ium, 3,4,5-trimethyl-, bromide	1.28
19.83	Trisulfide, di-2-propenyl	1.25
20.78	Cyclopentene, 3-methyl-3-(trimethylsilyl)acetyl-	0.71
21.72	Eugenol	0.53

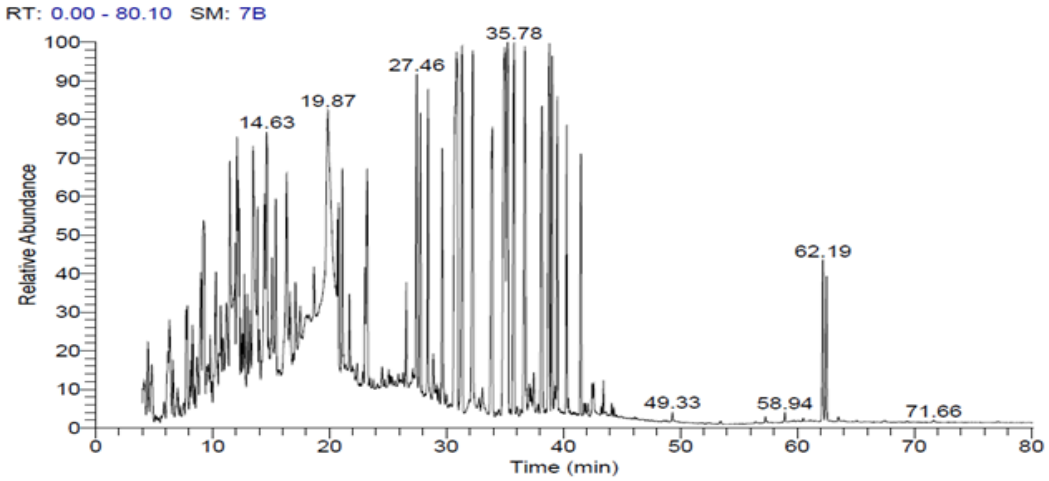

Fig. 3: GC chromatogram of garlic oil**.**

Table 6: Chemical composition of the peppermint essential oil by Gass Chromatography Mass Spectrophotometry.

RT	Compound Name	Area%
6.03	2-Hexenal, (E)-	0.94
8.24	Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl	3.46
8.85	1-Octen-3-ol	2.18
9.10	α-Phellandrene	3.83
9.18	α-Pinene	2.42
9.28	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)	1.57
9.74	3-Carene	0.72
10.22	D-Limonene	2.84
10.57	Limonene	1.53
10.57	p-Menth-8-en-1-ol, acetate	1.35
10.94	p-Mentha-1,4(8)-diene	0.96
10.69	Eucalyptol	1.86
10.87	1-Octanol	1.02
11.12	1-Nonen-3-ol	0.55
11.20	4-Isopropyl-1-methyl-2-cyclohexen-1-ol	0.59
11.57	Undecane	0.54
12.57	cis-p-Menth-2,8-dienol	0.75
14.10	p-Menth-3-en-1-ol	1.98
14.52	p-Menth-1-en-4-ol	1.84
17.20	p-Mentha-6,8-dien-2-one (carvone)	5.47
20.47	p-Menth-8-en-2-ol	4.62
23.22	Humulen-(v1)	7.21
24.52	Caryophyllene	5.09
26.88	β-Elemene	1.99
30.90	Cubenol	0.78

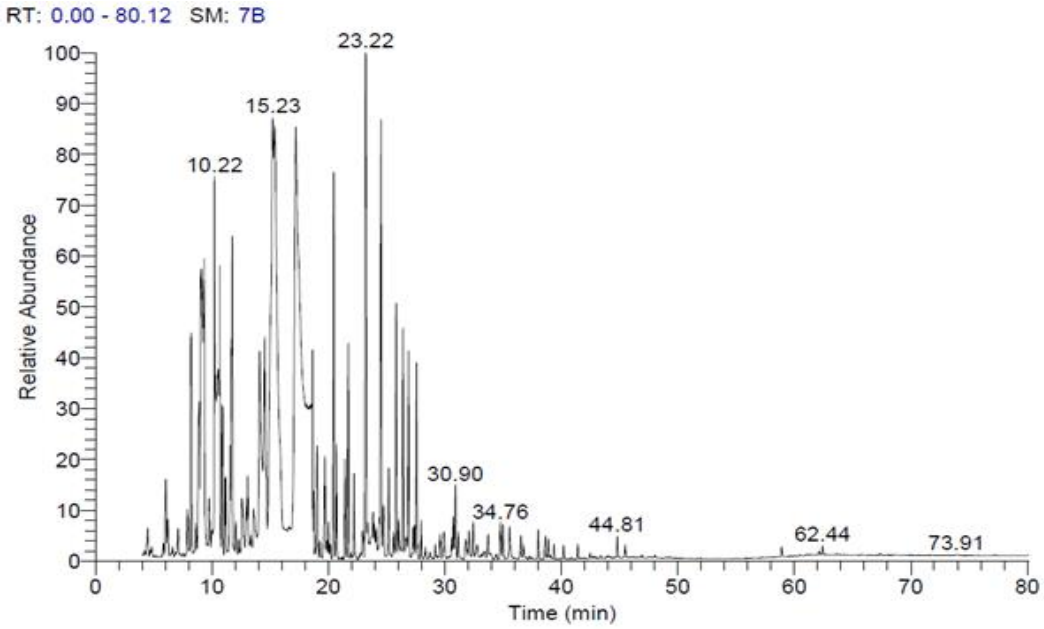

Fig. 4: GC chromatogram of peppermint oil**.**

Identification of *Candida* isolates

Based on the results of the different phenotypic tests used for the identification of the different *Candida species*, the predominant isolated species was C. *albicans* (25 isolates, 50%) followed by *C. glabrata* (22 isolates, 44%) and *C. krusei* (3 isolates, 6%).

Antifungal susceptibility of *Candida* isolates

A) Determination of MIC of clotrimazole drug and essential oils against *Candida* isolates

In this study, the *in vitro* susceptibility of isolated *Candida* species to tested essential oils and clotrimazole (used as standard) was determined by estimating their MICs in µg/ml (expressed as Mean ± SD) by the broth micro-dilution method. For different candida species, the MIC values of tested EOs were significantly lower than clotrimazole (p value <0.05 for all). **Table (7)**.

Table 7: MIC values of essential oils in comparison to clotrimazole drug against *Candida* species.

	MIC (µg/ml)	**EOs**	**MIC (µg/ml)**	**P. value**
C. albicans	**Clotrimazole =** 73.75±116.836	**Basil oil**	2.93±2.17	<0.001
		Clove oil	1.68±1.45	<0.001
		Garlic oil	30.63±16.86	<0.001
		Peppermint	30.94±18.66	<0.001
C. glabrata	**Clotrimazole =** 80.966±144.831	**Basil oil**	1.73±1.53	<0.001
		Clove oil	1.78±1.53	<0.001
		Garlic oil	6.21±5.86	<0.001
		Peppermint	1.78±1.53	<0.001
C. krusei	**Clotrimazole =** 5.244±2.224	**Basil oil**	0.98±0	0.034
		Clove oil	0.98±0	0.034
		Garlic oil	1.63±0.56	0.043
		Peppermint	1.3±0.56	0.043

MIC values (µg/ml) are expressed as Mean ± SD, P value ≤0.05 was considered statistically significant.

B) By disc diffusion method

All tested candida species showed the highest sensitivity to clotrimazole, *C. albicans* (80%), *C. glabrata* (81.82%) and*C. krusie(100%),* while the highest resistance was to nystatin. **Table (8)**.

Detection the effects of tested Essential Oils on *Candida* isolates

Effects of tested Essential Oils on *C. albicans* transition from blastospore to hyphal form.

Both basil oil and Clove oil suppressed *C. albicans* transition from blastospore to hyphal form, in contrast garlic oil and peppermint oil did not affect candida transition, compared to the control. **Fig. (5)**

Table 8: Antifungal sensitivity pattern of different *Candida* species by the disc diffusion method.

Species (No.)	Antifungal	Susceptible		Intermediate		Resistant	
		N0.	%	N0.	%	N0.	%
C. albicans (25)	Clotrimazole (10µg)	20	80	5	20	0	0
	Voriconazole (1 µg)	1	4	0	0	24	96
	Nystatin (100U)	0	0	1	4	24	96
	Amphotericin B (100U)	18	72	0	0	7	28
	Itraconazole (10 µg)	4	16	15	60	6	24
C. glabrata (22)	Clotrimazole (10µg)	18	81.82	2	9.09	2	9.09
	Voriconazole (1 µg)	16	72.73	3	13.64	3	13.64
	Nystatin (100U)	0	0	4	18.19	18	81.82
	Amphotericin B (100U)	18	81.82	0	0	4	18.19
	Itraconazole (10 µg)	6	27.27	8	36.36	8	36.36
C.krusie (3)	Clotrimazole (10 µg)	3	100	0	0	0	0
	Voriconazole (1 µg)	3	100	0	0	0	0
	Nystatin (100U)	0	0	0	0	3	100
	Amphotericin B (100U)	3	100	0	0	0	0
	Itraconazole (10 µg)	0	0	0	0	3	100

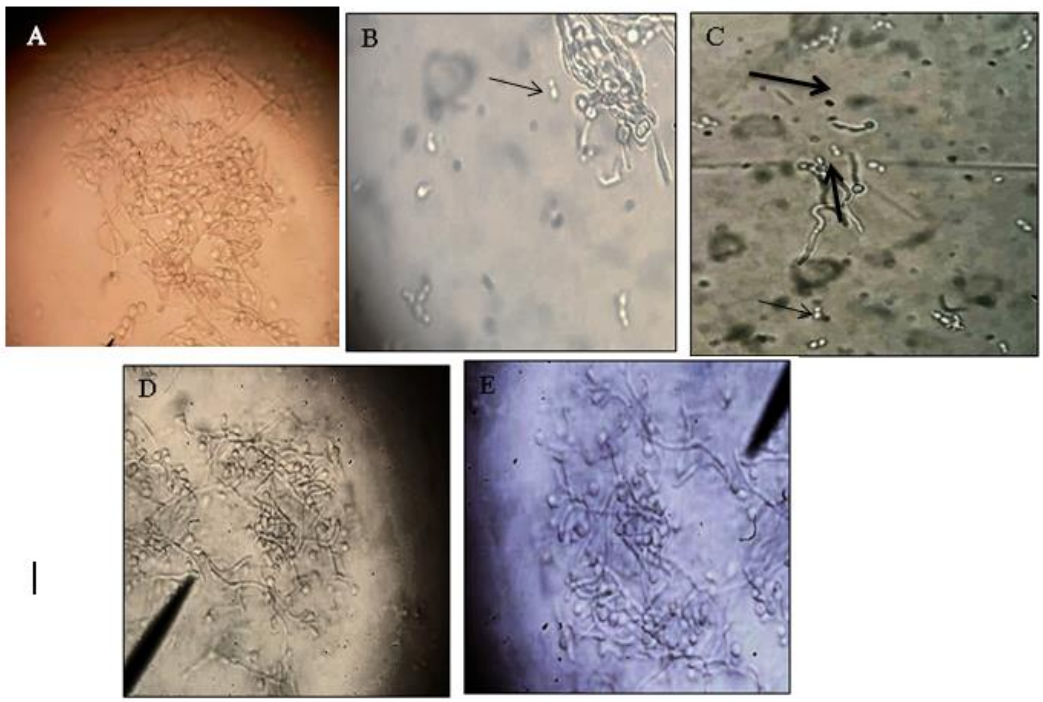

Fig. 5: Detection the effect of the essential oils on *Candida albicans* transition from blastospore to hyphal form: **A)** showing Untreated *C. albicans* with yeast hyphal form, **B) & C)** Showing *C. albicans* treated with basil oil and clove oil respectively that suppressed *Candida* transition from blastospore to hyphal form, **D) &E)** Showing *C. albicans* treated with garlic oil and peppermint oil respectively which did not affect candida transition, compared to the control.

Effect of clotrimazole and essential oils on *Candida* species ultrastructure by scanning electron microscope

By using SEM for imaging of different *Candida* species, the untreated yeast cells revealed typical appearance displaying characteristic bud scars. Following the addition of EO, the external morphology of the cells did not appear as smooth as that of the untreated cells, which indicates a possible loss of cytosolic volume. EO distorted the cell wall surface and made pores in the cells. These distorted cell features were comparable to those observed in the presence of clotrimazole **(Fig.s 6-8).**

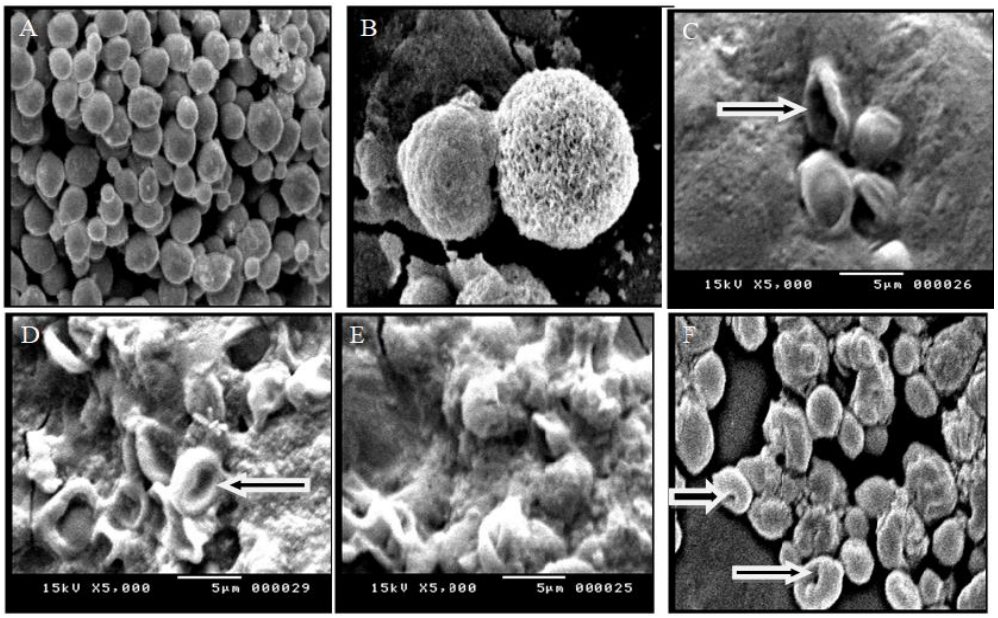

Fig. 6: Effects of clotrimazole and essential oils on *C. albicans* ultrastructure; A) Untreated cells (typical yeast cells displaying characteristic buds, no hyphal development was observed), **B)** Treated with clotrimazole (cells become large in size and have pores), **C)** Treated with basil oil (cells show lysis and have pores), **D)** Treated with clove oil (cells show distortion and have pores), **E)** Treated with garlic oil (cells show distortion), **F)** Treated with peppermint oil (cells show pores).

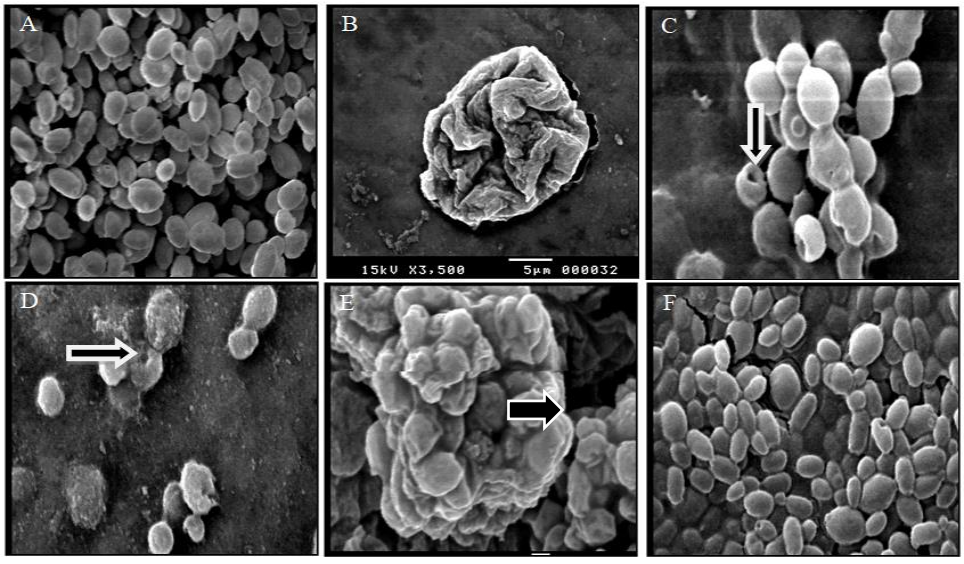

Fig. 7: Effects of clotrimazole and essential oils on *C. glabrata* ultrastructure; A) Untreated cells (typical yeast cells displaying characteristic bud scars. **B)** Treated with clotrimazole (cells become wrinkly), **C)** Treated with basil oil (cells show pores and cell lysis), **D)** treated with clove oil (cell lysis and formation of pores on cell surface), **E)** Treated with garlic oil (disrupted cells), **F)** Treated with peppermint oil (cells show pores).

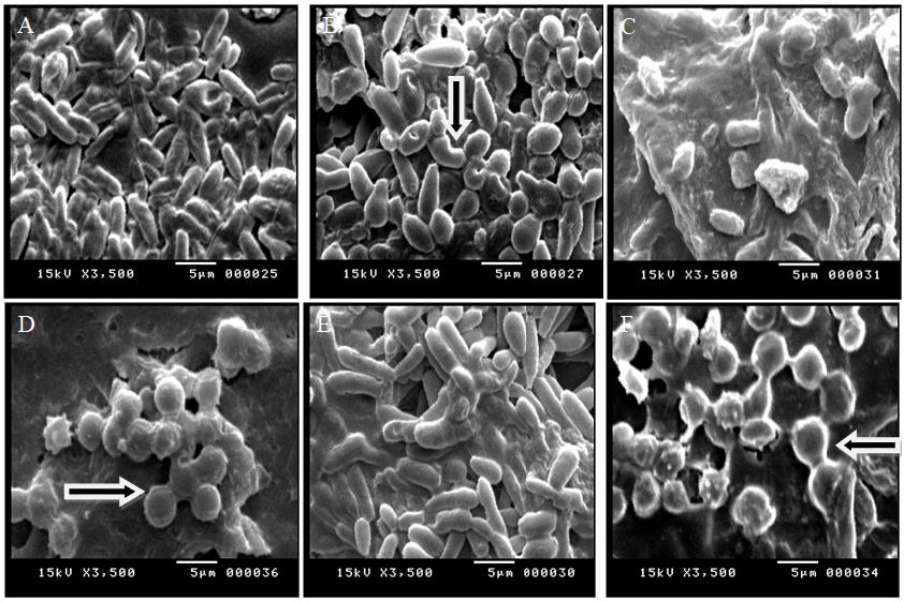

Fig. 8: Effects of clotrimazole and essential oils on C. krusei ultrastructure; A) untreated cells (small elongated ovoid budding yeast cells). B) Treated with clotrimazole (show pores in cells and curved cells), C) Treated with basil oil (show cell lysis), D) Treated with clove oil (show rounding cells and cell lysis), E) Treated with garlic oil (show distortion of cells), f) Treated with peppermint oil (show cell lysis and rounding of cells).

Detection of biofilm formation by *Candida* species by microtiter plate method.

From 50 isolates of *Candida* species 18 (36%) isolates formed biofilm; 11 isolates of *C. albicans*, 4 isolates of *C. glabrata* and the 3 isolates of C. *krusei*. The remaining 32 (64%) isolates were non-biofilm producers. different candida species displayed variable degree of biofilm formation as demonstrated in **table (12)** and **fig. (9).**

Table 12: Results of detection of biofilm formation by *Candida* species.

	C. albicans **(N=25)**	***C. glabrata*** **(N=22)**	***C. krusei*** **(N=3)**	**Total (N= 50)**
Strong biofilm producer	5	1	1	7 (14%)
Moderate biofilm producer	5	1	1	7 (14%)
Weak biofilm producer	1	2	1	4 (8%)
Non-biofilm producer	14	18	0	32 (64%)

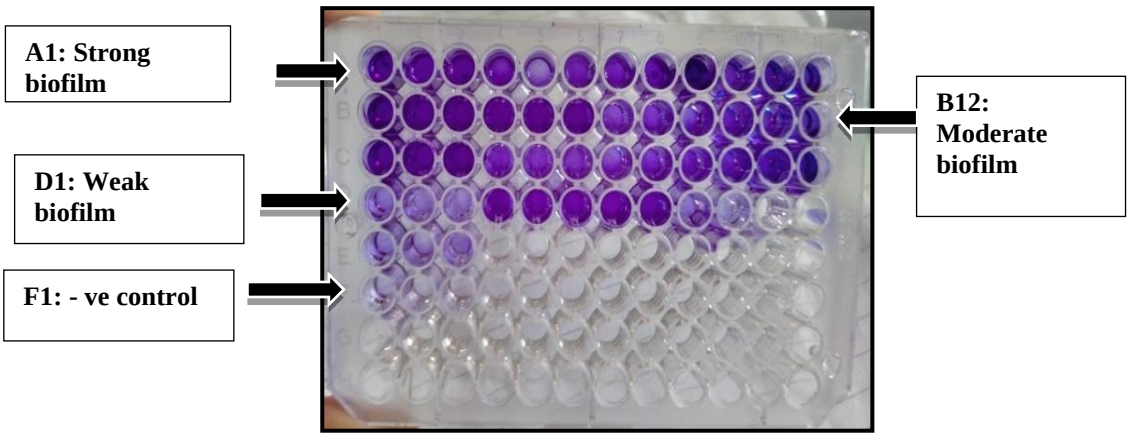

Fig. 9: Detection of biofilm formation by *Candida* species by microtiter plate method.

Effect of clotrimazole drug and essential oils on *Candida* species biofilm formation.

A dose-dependent reduction in biofilm formation was observed when different concentrations of clotrimazole and essential oils were added to the different form of *Candida* biofilm (strong, moderate and weak) **(Fig. 10-12)**. Moreover, percent of inhibition of biofilm formation of clotrimazole and essential oils treated *Candida* isolates (in comparison to untreated controls) were calculated **(table 19).**

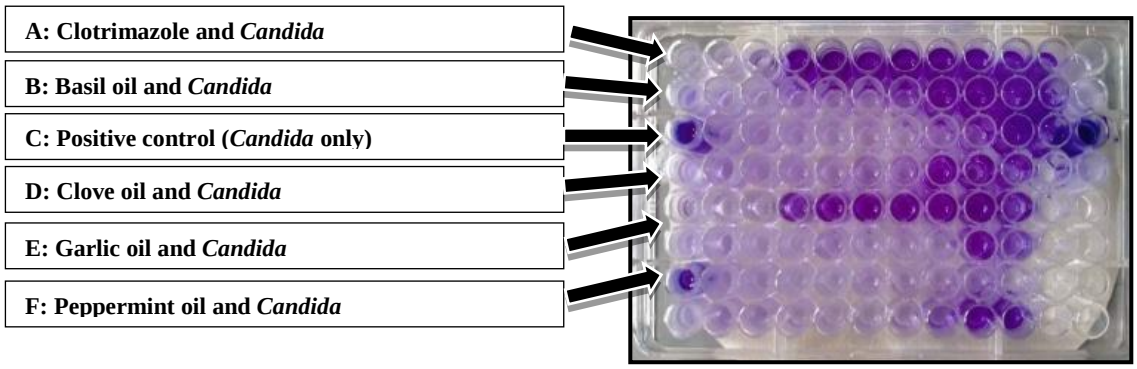

Fig. 10: Inhibition of strong biofilm by different concentrations of clotrimazole and essential oils.

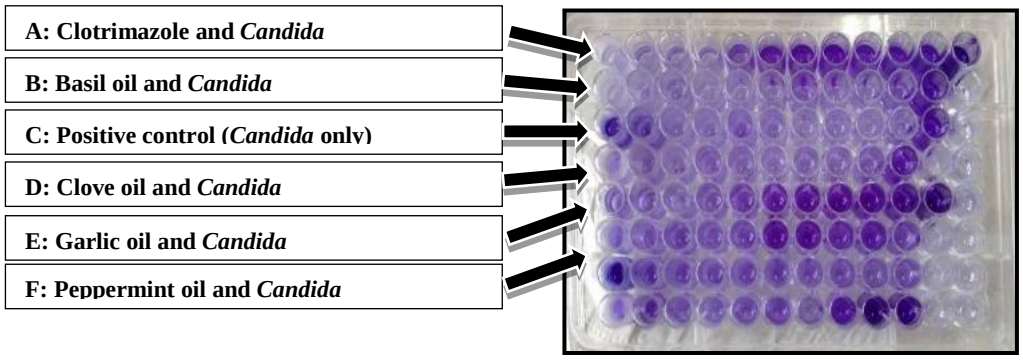

Fig. 11: Inhibition of moderate biofilm by different concentrations of clotrimazole and essential oils.

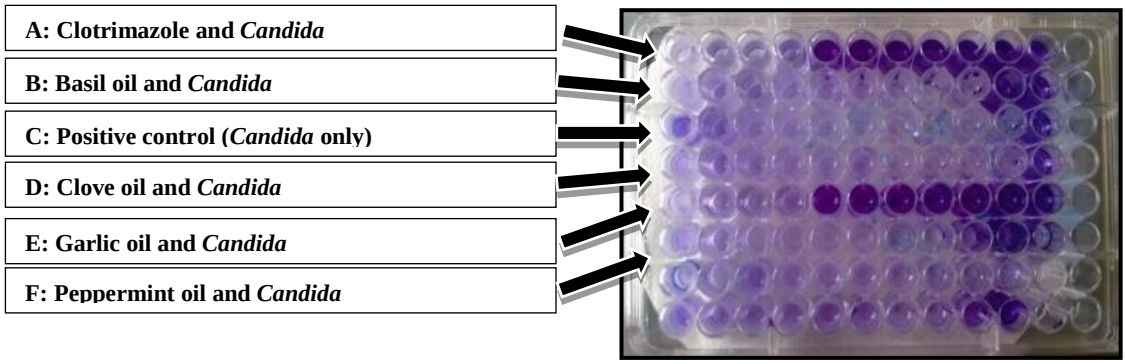

Fig. 12: Inhibition of weak biofilm by different concentrations of clotrimazole and essential oil.

Table 13: Percent of biofilm inhibition in clotrimazole and essential oils treated *Candida* isolates (in comparison to untreated control).

	C. albicans	***C. glabrata***	***C. krusei***
Clotrimazole	47.96%	66.05%	66.07%
Basil oil	67.99%	66.64%	75.25%
Clove oil	67.83%	58.63%	68.73%
Garlic oil	55.68 %	55.82%	74.75%
Peppermint oil	56.54%	45.79%	60.56%

Effect of clotrimazole, basil oil and clove oil on *C. albicans* Genes *(HWP1, ALS3* and *SAP3)* Activation/Repression

Quantitative RT–PCR experiments revealed *HWP1* gene expression upregulation when clotrimazole and basil oil added to *Candida albicans* culture, but *HWP1* gene expression downregulated when clove oil added to *C.albicans* culture **Fig..13**. *ALS3* and *SAP3* gene expression downregulated when clotrimazole, basil and clove oil added to *Candida albicans* culture **Fig. (14) & Fig. (15)**. The repressive effect of clotrimazole and clove oil on *ALS3* gene expression was higher than basil (63.4%) and (70%) respectively as shown in **Fig. (32)**. The repressive effect of clove oil on *SAP3* gene expression was higher than basil oil (93%) and clotrimazole (93.3%).

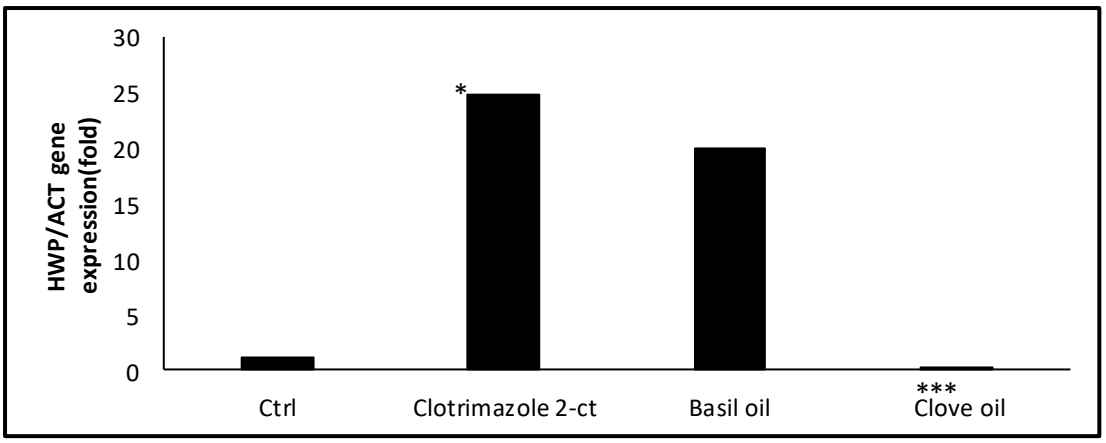

Fig. 13: Effect of clotrimazole, basil and clove oil on *HWP*1 gene expression. * $p < 0.05$, ***$p<0.001$.

Fig. 14: Effect of clotrimazole, basil and clove oil on *ALS3* gene expression. *$p < 0.05$

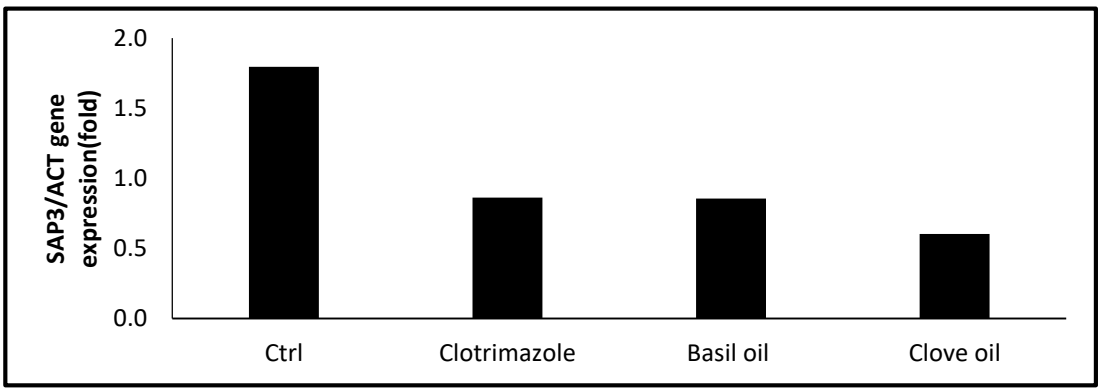

Fig. 15: Effect of clotrimazole, basil and clove oil on *SAP3* gene expression.

Discussion

There is observed marked increase in deaths from invasive fungal infections with current global estimates of 1,000,000 cases or more each year. Among these fungal opportunists are different species of *Candida albicans*[26].

Drug-resistant pathogens are becoming an increasing threat to human health leading to an increased incidence of debilitating and fatal infections. Plants and their extracts have long been utilized to treat a variety of ailments, including infectious disease. Many plant essential oils have antimicrobial properties that are known to inhibit viruses, bacteria, and fungi. They are readily available and generally inexpensive, increasing their accessibility as an attractive reservoir for antimicrobial treatments[27].

The chemical composition of essential oils relies on the type of extraction methods applied. Also, the percent of the main components of essential oil vary by many environmental factors namely rainfall, sunshine rate, light levels and the plant cycle, mainly in relation to flowering. Moreover, some minor chemical constituents exist only in specific origin. So that the constituents of medicinal plants' essential oils have to be carefully identified before performance of any further experiments[28&29].

In this study, the essential oils (basil, clove, garlic and peppermint) were extracted by the distillation method using the Clevenger type apparatus and their main constituents were identified by GCMS.

In the present study the major components of basil oil were bornyl acetate (6.74%), α – Farnesene (4.10%), Cubenol (3.48%), Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl) (3.26%), α-Cubebene (2.93%) and Eugenol (2.68%). While previous study found that the main components of *Ocimum basilicum* oil were methyl eugenol (78.02%), α-cubebene (6.17%), nerol (0.83%) and ε-muurolene (0.74%) [30]

For clove oil; the major components of clove oil were eugenol (16.94%), α-elemen (11.91%), isoeugenol (11.36%), caryophyllene (7.85%), cadina-1(10),4-diene (5.49%) and isocaryophyllene (3.55%). In previous study revealed significant differences between clove oil from two sources (Java and Manado; Indonesia). Both origins had same major constituents but different percentage compositions. Java clove contained eugenol (55.60 %), caryophyllene (14.84 %), eugenyl acetate (20.54 %), and α-humulene (2.75 %). While, in Manado clove, the composition were eugenol (74.64 %), caryophyllene (12.79 %), eugenyl acetate (8.70 %), and α-humulene (1.53 %)[31] .

Wongsawan, *et al*.[33] showed that eugenol was the major component (97.76%) but Caryophyllene (1.17%), gamma-humulene (0.39%) and caryophyllene oxide (0.78%) were the minor components [32] while Ratri et al. reported that eugenol and eugenyl acetate were around (85.01%) and (13.06%) respectively. These results indicated that the geographic origin and the growing conditions may affect the chemical constituents in clove .

In the present study the major components of garlic oil were dimethyl trisulfide (2.43%), trisulfide, methyl 2-propenyl (1.43%), tetrasulfide, di-2-propenyl (1.61%), 1,2-Dithiol-1-ium, 3,4,5-trimethyl-, bromide (1.28%), trisulfide, di-2-propenyl (1.25%), Dodecane (1.25%) and 2-Vinyl-1,3-dithiane (1.22%). Our results match greatly with Satyal et al. that analyzed the constituents of garlic EO and found that it were dominated by allyl polysulfides, including diallyl sulfide (1.9–9.5%), diallyl disulfide (20.8–27.9%), diallyl trisulfide (16.8–33.4%), allyl methyl disulfide (4.4–8.3%), and allyl methyl trisulfide (14.5–19.2%) [34] .Also, the studies reported by Herrera-Calderon et al. showed that diallyl trisulphide was the main component (44.21%) followed by diallyl sulphide (22.08%) [35] .

In this study we concluded that the major components of peppermint oil were humulene (7.21%), p-mentha-6,8-dien-2-one (carvone) (5.47%), caryophyllene (5.09%), p-menth-8-en-2-ol (4.62%), α-phellandrene (3.83%) and bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl (3.46%). These findings were similar to Mackled et al. who found that peppermint oil contains a percent of caryophyllene (4.82%) and α-Pinene (2.25%)[36] . However, the study reported by Camele, *et al*.[37]showed that α-humulene (0.07%) and caryophyllene (2.96%) were minor components .This results matches with AL-MIJALLI, *et al*.[38]who reported that the major components of peppermint in Azrou region (northen Morrocco) was carvone (70.25%) as the most dominant compound identified from 17 compounds while in Ouezzane (northen Morrocco) found that the

major component were menthol (43.32%) and menthone (29.4%) as the most dominant compounds from 22 compound .

In this study out of the 50 yeast isolates obtained by culture on SDA, 25 (50%) isolates were identified as *C. albicans*. The remaining 25 (50%) isolates were non-*albicans Candida* species; 22 (44%) isolates were *C. glabrata* and 3 (6%) isolates were *C. krusei* . These results were matched with Aslani, et al.[39] study that isolated yeasts from the oral cavity of cancer patients and found that *C. albicans* was the most common species (50.6%), followed by *C. glabrata* (24.7%), *C. krusei* (9.9%) in addition to other *Candida* species. A recent Egyptian study reported that non-*albicans Candida* spp. *(C. tropicalis* and *C. parapsilosis*) was common (38.1%) as *C. albicans* (61.9%) in acute myeloid leukemia patients with invasive fungal infections[40].

In this study, the *in vitro* susceptibility of isolated *Candida* species to tested essential oils and clotrimazole drug (used as standard) was determined by estimating their MICs in µg/ml (expressed as Mean ± SD) by the broth micro-dilution method and by evaluating their diameters of inhibition zones (expressed as Mean ± SD) by well diffusion assay. However, the use of agar diffusion technique produces results that sometimes show little antimicrobial activity, but the same essential oil is observed to have high activity when using the vapor phase diffusion test. This phenomenon could be explained by the diffusion coefficient and water solubility of essential oils[41].

For *C. albicans*, the MIC values of tested EOs {Basil oil (2.93±2.17), Clove oil (1.68±1.45), Garlic oil (30.63±16.86) and Peppermint (30.94±18.66)} were significantly lower than clotrimazole (73.75±116.836) (p value <0.001 for all). When comparing these EOs to each other's, MIC value of clove oil was significantly the lowest compared to basil, garlic and peppermint oils (p values; 0.007, <0.001 and <0.001 respectively) while MIC value of Peppermint oil was significantly the highest compared to basil and clove oils (p values; <0.001 for both) and insignificantly higher compared to Garlic oil (p value =0.950).

For *C. glabrata*, the MIC values of tested essential oils {basil oil (1.73±1.53), clove oil (1.78±1.53), garlic oil (6.21±5.86) and peppermint (1.78±1.53)} were significantly lower than clotrimazole (80.966±144.831) (p value <0.001 for all).

So the tested essential oils have lower antifungal effect on *C. glabrata* isolates in comparison to clotimazole. However; these oils when compared to each other's, clove oil was the best. Our results differ from Tullio, *et al*.[42] study that reported non-negligible activity of Peppermint oil against *C. glabrata* that are often resistant to conventional drugs, Pinto, *et al*.[43]study that concluded antifungal properties of clove oil against *C. glabrata* and Vieira, *et al*.[44]who found marked activity of *Ocimum* (Basil) essential oils against the *Candida* species including *C. glabrata* due to the presence of effective antifungal compounds as eugenol and anethole .

For *C. krusei*, the MIC values of tested essential oils {basil oil (0.98±0), clove oil (0.98±0), garlic oil (1.63±0.56) and peppermint (1.3±0.56)} were significantly lower than clotrimazole (5.244±2.224) (p values; 0.034, 0.034, 0.043 and 0.043 respectively).

In our study, the in vitro antifungal susceptibility testing was performed by disc diffusion method for selected azoles (clotrimazole, voriconazole and Itraconazole) and polyene antifungal agents (amphotericin B and nystatin).

C. albicans isolates (N= 25) showed the highest sensitivity to clotrimazole and amphotericin B (20 (80%) and 18 (72%) isolates respectively) while the highest resistance was to voriconazole and nystatin (24 (96%) isolates for both). *C. glabrata* isolates (N=22) showed the highest sensitivity to clotrimazole, voriconazole and amphotericin B (18 (81.82%), 16 (72.73%) and 18 (81.82%) isolates respectively) while the highest resistance was to nystatin (18 (81.82%) isolates). All *C. krusie* isolates (N= 3) were sensitive to clotrimazole, voriconazole and amphotericin B, while all these isolates were resistant to nystatin and itraconazole. In other words, the highest sensitivity of all *Candida* isolates was to clotrimazole and amphotericin B while the highest resistance was to nystatin.

The results of our study were in agree with Khadka et al., and Tamai *et al*.[45&46] who reported that sensitivity of *Candida* species to clotrimazole was (82% and 70% respectively). Also our results agree with Marak & Dhanashree[3] who reported that the sensitivity of *Candida albicans*, *Candida glabrata*, and *Candida krusei* isolates investigated in their study to Amphotricin B was 100%. Khan, et al.[47] study reported marked resistance of *C.*

albicans, *C. glabrata* and *C. krusei* isolates to nystatin (55.5%, 68.7% and 50% respectively) that matches greatly with results of our study . However, Khan, *et al.*[47] study also reported marked resistance of *C. albicans*, *C. glabrata* and *C. krusei* isolates to clotrimazole (60%, 62.5%, and 55.5% respectively) that disagree with the results of our study a matter that can be explained on basis of epidemiological differences in distribution of resistant strains .

This study investigated the effect of essential oils on the ability of germ tube formation by *C. albicans* as a predictor of its ability for transition from blastospore form to hyphal (invasive) phenotype. We concluded that germ tube formation was indeed inhibited following the addition of basil and clove oils to the cultures of *C. albicans* (compared to the control cultures). These results match with Sani, *et al.*[48] study that reported the inhibitory effects of methyl chavicol (one of the active components present in basil oil) on germ tube induction and Shahina, *et al.*[49] study that investigated the effect of clove oil on virulence of *C. albicans* and declared that eugenol (a major component of clove oil) is an effective disruptor of membrane integrity. Membrane compromise initiates a series of events that impact a wide range of processes vital for infection, including the secretion of hydrolytic enzymes, endocytosis and hyphal morphogenesis.

Although this study concluded that germ tube formation was not affected by garlic and peppermint oils, many other studies reported its marked inhibition by these oils as Benzaid, *et al.*[23] who found that Peppermint oil and even its volatile vapor could inhibit germ tube formation by cultures of *C. albicans* and Yousuf, *et al.*[50] who found that Garlic-derived allyl sulphides have an inhibitory effect on *C. albicans* germ tube formation. However, this observation notably needs more studies to explain it (as the possibility of acquired resistance, the need for special extraction method of essential oil or the need for special storage conditions).

Our current study investigated the possible mechanism of antifungal properties of tested essential oils in comparison to coltrimazole (as a reference) at the cell level by examination of cell ultrastructure changes after treatment with these oils. We found that fungal cell treatment with essential oils produced cell ultrastructure changes that are comparable to/ or even more manifest than clotrimazole (cell distortion, cell size change, membrane pore formation and cell lysis). Our results match with Bona, *et al.*[51] study that concluded that Mint and Basil essential oils produced cell damage of *C. albicans* more than clotrimazole as detected by SEM, Latifah-Munirah, *et al.*[52] study found that eugenol, a phenylpropene compound extracted from cloves, caused marked disruption of the cell wall of *C. albicans* and Ezeorba, *et al.*[53] who observed that garlic essential oil could enter *C. albicans'* cellular membrane as well as the membranes of organelles like mitochondria, causing organelle damage and cell death, as revealed by TEM . To our knowledge, no other studies investigated the effects of these tested essential oils on ultrastructure of non-albicans *Candida* species, a point that marks the novelty of our study.

Our study screened the *Candida* isolates for biofilm production by microtiter plate method. Out of the 50 isolates of *Candida* species; 18 (36%) isolates formed biofilm (11 isolates of *C. albicans*, 4 isolates of *C. glabrata* and the 3 isolates of C. *krusei*). Out of these 18 biofilm producing *Candida* isolates, 7 (14%) isolates were strong biofilm producer, 7 (14%) isolates were moderate biofilm producer and 4 (8%) isolates were weak biofilm producers. The remaining 32 (64%) isolates were non-biofilm producers. Our results match with Hasan, *et al*. and Tellapragada, *et al*.[54&55] studies who concluded higher biofilm production in *C. albicans* as compared to non-albicans *Candida*.

Our study observed a dose-dependent reduction in biofilm formation (that is comparable to Clotrimazole drug) when different concentrations of essential oils were added to the growing biofilms of *Candida* species. Moreover, percent of inhibition of biofilm formation of essential oils treated *Candida* isolates (ranged from 45.79% to 75.25%) were comparable to clotrimazole treated *Candida* isolates (ranged from 47.96% to 66.07%). These findings match greatly with Rajkowska, *et al*.[56] who declared that in the presence of clove oil, 68.4–84.2% of the tested yeasts showed a statistically significant reduction in biofilm formation so clove essential oil can be efficiently used in the prevention of surfaces colonization by *Candida spp* , Cardoso, *et al*.[57] who concluded that biofilm formation was inhibited in *C. albicans* strains by basil essential oil , Benzaid, *et al*.[23]

study data demonstrated the efficacy of *Mentha piperita* EO against the formation of *C. albicans* biofilms and Pereira, *et al*.[58] results that showed that garlic essential oil caused a reduction of progressive increase in the mass of biofilms produced from the of all tested *C. albicans.*

HWP1 is a well-characterized *Candida albicans* cell surface protein, expressed only on hyphae, that mediates tight binding to epithelial cells. *ALS* (agglutinin-like sequence) adhesins, required for host adhesion and secreted aspartyl proteases (*SAPs*) allow for the degradation of host barriers and the invasion of surrounding tissue[8] .

Results of our study showed that *HWP1* gene expression was upregulated when clotrimazole and basil oil were added to *Candida albicans* culture and downregulated when clove oil was added to *candida albicans* culture. Maras, *et al*.[59] study of three genes correlated with virulence, *CDR1, CDR2* and *HWP1*, in clinical *C. albicans* isolates showed that gene hyper-expression conferred a less aggressive phenotype . On the contrary, in other isolates a decreased expression of *CDR1, CDR2* and *HWP1* may be consistent with the less aggressive performance. This altered gene expressions might directly influence *Candida* virulence or might be an epiphenomenon of a vaster rearrangement occurred in these strains during the challenge with the host's environment. This result suggested that there are other genes controlling the biofilm development and virulence of *Candida albicans* Sakkarin Lethongkam, *et al*.[60] reported that bio silver nanoparticles of aqueous *Eucalyptus camalulensis* downregulated the expression level of all biofilm related genes (*ALS3, ECE1, EFG1,TEC1,ZAP1,PLB2, LIP9* and *SAP4*) .Similar result showed by Chatrath.A, *et al*.[61]who reported that citral upregulated ERG11 and CYT450 genes and Thymol upregulated CNB1 and SOD1 genes.

ALS3 and *SAP3* gene expression were downregulated when clotrimazole, basil and clove oil were added to *Candida albicans* culture. The inhibitory effect of clotrimazole on *ALS3* gene expression was higher than basil (63.4%) and clove oil (70%). The inhibitory effect of clove oil on *SAP3* gene expression was higher than basil oil (93%) and clotrimazole (93.3%). These results match with Man, *et al*.[62] that proved a down regulatory effect of basil and clove essential oils on *ALS3* gene expression. To our knowledge, no other studies investigated the effect of basil and clove EOs on *SAP3* gene expression in *C. albicans*, a point that marks the novelty of our study.

Conclusion

This study concluded that clove oil is the best essential oil among other EOs that inhibited growth, transition and biofilm formation by lowest MIC and downregulated *HWP1, AlS3* and *SAP3* gene expression that responsible for resistance of *Candida albicans*.

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عنوان تأثير الزيوت الطياره لنباتات مختارة على نموعزلات اكلينيكية من المبيضات والانتقال وتشكيل الاغشيه الحيويه

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لقد أدي إرتفاع ظهور مقاومة المبيضات لمضادات الفطريات إلى إهتمام كبير بتطوير عقاقير جديدة مضادة للفطريات باستخدام الأدوية الطبيعية مثل الزيوت الطياره العطرية النباتية . ولقد هدفت هذه الرسالة إلي تقييم مدي تأثيرالزيوت الطياره العطريه (الريحان،القرنفل،الثوم، النعناع) على نمو أنواع المبيضات المختلفة، والبنية التحتية لخلاياها ، وتشكيلها للأغشية الحيوية وكذلك على التعبير الجيني لبعض جينات الضرواة (HWP1, ALS3, SAP3).

ولقد قمنا بإعداد زيوت أساسية لبعض النباتات الطبية (الريحان ، والقرنفل ، والثوم ، والنعناع) وقد تم عزل ٥٠عزلة من المبيضات من مرضى داء المبيضات الفموي وقد اشتملت المبيضات المعزولة علي ثلاثة انواع من أنواع المبيضات: المبيضات البيضاء والمبيضات الجرداء و مبيضات الكروزي. و تم إجراء اختبار الحساسية للمضادات الفطرية التابعة لمجموعة الأزولات (كلوتريمازول وفوريكونازول وإيتراكونازول) وأخري تابعة لمجموعةالبوليينات (أمفوتيريسين ب ونيستاتين) بإستخدام اختبار الحد الأدنى من التركيز المثبط وطريقة الإنتشار القرصي .ولقد بحثت دراستنا في مدي تأثير الزيوت العطرية على قدرة تكوين الأنبوب الجرثومي بواسطة المبيضات البيضاءكمؤشر لقدرتها على الانتقال من شكل الأبواغ المتفجرة إلى النمط الظاهري (الغازي). كما بحثت دراستنا الحاليه في تاثير الزيوت الأساسية المختبرة على تغييرات البنية التحتية لخلايا المبيضات باستخدام الميكروسكوب الالكتروني وقد تم مقارنة هذا التأثير بتأثير الكلوتريمازول (كمرجع) على تغييرات البنية التحتية لخلايا المبيضات، كما تم فحص قدرة المبيضات علي تكوين البيوفيلم بواسطة استخدام أجار الكونغو الأحمر وتم تأكيده بواسطة لوحة ميكروتيتر. وبحثت دراستنا في النسبة المئوية لتناقص تكوين الأغشية الحيوية في عزلات المبيضات المعالجة بالكلوتريمازول والزيوت الأساسية (مقارنة بالمبيضات الغير معالجة) .ولقد تناولت هذه الدراسة أيضا تأثير الكلوتريمازول وزيت الريحان والقرنفل على التعبير الجيني HWP، SAP3، ALS3بإستخدام اختبار تفاعل البلمرة المتسلسل الكمي .

ولقد أظهرت النتائج أن جميع عزلات المبيضات كانت لها حساسية عالية للكلوتريمازول والأمفوتيريسين بينما كان النيستاتين الأقل تأثيرا بين مضادات الفطريات المختبرة. و بالنسبة للزيوت المختبرة كان تأثيرها متباينا علي أنواع المبيضات المختلفة فكان الحد الأدنى من التركيز المثبط لزيت القرنفل هي الأدنى بشكل ملحوظ ضد المبيضات المبيضة مقارنة بزيوت الريحان والثوم والنعناع.) بينما كان الحد الأدنى من التركيز المثبط لزيت الريحان هو الأدني بشكل واضح ضد المبيضات الجرداء مقارنة بزيوت القرنفل والثوم والنعناع، وفيما يخص مبيضات الكروزي كانت قيم الحد الأدنى من التركيز المثبط لزيت الريحان وزيت القرنفل (٠,٩٨،٠,٩٨) علي التوالي . ، وزيت الثوم (١,٦٣ ± ٠,٥٦) والنعناع (١,٣ ± ٠,٥٦)} أقل بكثير من كلوتريمازول (٥,٢٤٤) ± ٢,٢٢٤) (قيم p ؛ ٠,٠٣٤ و ٠,٠٣٤ و ٠,٠٤٣ و ٠,٠٤٣ على التوالي).

وفيما يخص تأثير الزيوت العطرية على قدرة تكوين الأنبوب الجرثومي بواسطة المبيضات البيضاء خلصنا إلى أن تكوين أنبوب الجرثومية قد تم تثبيطه بالفعل بعد إضافة زيوت الريحان والقرنفل إلى مزارع المبيضات المبيضة (مقارنة بمزارع التحكم). ولقد وجدنا أن معالجة الخلايا الفطرية بالزيوت الأساسية أنتجت تغييرات في البنية التحتية للخلايا مماثلة / أو تزيد عن تأثير الكلوتريمازول .كما أظهرت نتائج لوحة ميكروتيتر أنه من بين ٥٠ عزلة من أنواع الكانديدا ، شكلت ١٨ عزلة (٣٦٪) غشاء حيوي. ٧ (١٤٪) منتج قوي للأغشية الحيوية ، ٧ (١٤٪) منتج متوسط للأغشية الحيوية و ٤ (٨٪) منتج ضعيف للأغشية الحيوية. بحثت دراستنا في النسبة المئوية لتناقص تكوين الأغشية الحيوية في عزلات المبيضات المعالجة بالكلوتريمازول والزيوت الأساسية (مقارنة بالضوابط غير المعالجة) وكان لزيت الريحان ضد المبيضات البيضاء والمبيضات الجرداء و مبيضات الكروزي أعلى نسبه (٦٧,٩٩ % ، ٦٦,٦٤ % ، ٧٥,٢٥ %) على التوالي مقارنة بالكلوترمازول وزيوت القرتفل والثوم والنعناع . التعبير الجيني للجينات *HWP1 ,SAP3, A LS3,* اصبح اقل عند إضافة زيت القرنفل الى مزرعة المبيضات البيضاء.