Anti fungal effect of some plant oils against some oral clinical isolates of *Candida albicans* in Lebanese community

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Abstract: Oils extracted from different medicinal plants (volatile and fixed) were screened for their activity against oral pathogen *Candida albicans* by agar well diffusion method. Minimum inhibitory concentrations (MICs) of oils against *Candida albicans* done by agar dilution method and minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFCs) data obtained by the broth micro dilution method. The results showed that the maximum antifungal activity was demonstrated by pine, tea tree, oreganum, thyme and clary sage oil as compared to nystatin as a control. While clove,eucalyptus ,cinnamon , lemon and lavender oil exhibited moderate inhibitory activity. Cotton ,aniseed and rose oil demonstrated low activity as compared to control. Mustard ,linseed, peppermint, sesame, fennel, caraway, cumin, cactus, castor, blackseed and almond oil did not show any antifungal activity. More over Time-Kill assay shows that *Candida albicans* will be almost killed after 6 hours of treatment with the promising oils. These results support the plant oils can be used to cure fungal infections and plant oils may have role as pharmaceutical.

Key words: plant essential oils, *Candida albicans*, antifungal activity, in-vitro studies.

Introduction

Many essential oils have been advocated for use in complementary medicine for bacterial and fungal infections including acne, vaginal candidiasis and oral thrush. However, few of the many claims of therapeutic efficacy have been validated adequately by either in-vitro testing or in-vivo clinical trials. (Citak et al, 2005)

Candida albicans is a yeast that occurs naturally in the body. The body's natural defenses normally keep yeast in check but, if there is an imbalance, the yeast can grow out of control .*Candida* thrives in warm, moist places such as the vagina, the mouth and between the folds of the skin. Infections occur when competing bacterial flora are eliminated by antibacterial antibiotics allowing this yeast to overgrow leading to various manifestation depending on the site such as oral candidiasis(thrush) and vaginal candidiasis**.** *Candida albicans* are less susceptible to azoles derivatives with increasing frequency. Nystatin and Ketoconazole, two important agents against human pathogenic fungi, have side effects as well as toxic effects. Thus there is the need for better, novel antifungal agents against infections by some fungi, especially Candida species.(Devkatte et al, 2005 and Mahdavi et al, 2009)

In the last two decades, some research has focused on using herbal components, which have fewer side effects. Meanwhile, extracting effective drug components from these herbs, such as herbal essential oils, which are used as antimicrobial, antiviral, and antifungal agents, is increasing. Plant-derived essential oils are natural, cheaper, and safer, thus; plant extracts are preferred in the cure of fungal infections. Some of these plants' essential oils are used as a remedy for headaches, arthritis, and also to treat skin discoloration, infectious, and parasitic diseases.(Chaieb et al.(2007). Antifungal in vitro susceptibility testing should provide useful information for selecting the most active drug against etiological agents. Several oils of plant origin have been used in ancient medicine against some infections in the world many years age. These compounds play essential roles in traditional medicine especially in developing countries. The present investigation examines the in-vitro susceptibility to *Candida spp* to a range of essentail oils. They will then be compared with anti-fungal drugs to find out their efficacy in the prevention and treatment of the diseases (Motsei et al.,2003)

Materials and Methods

Test organism : Five different strains of *Candida albicans* were used throughout the present work. They were isolated from oral samples of patients from Lebanese community and obtained from Elias Hrawi Governmental Hospital and were further identified as *Candida albicans* using the simplified identification method SIM key proposed by Deak (1986). They showed a wide variation in sensitivity towards the oils and nystatin. Therefore they were identified as different strains of *C.albicans* (n_1, n_2, n_3, n_4 and n_5). All the fungal isolates used throughout the present investigation were maintained on Sabouraud Dextrose agar slants folded with 25 % glycerol, and stored at 4^0C with regular transfer at monthly intervals.

Inoculum preparation for antifungal susceptibility tests

Inocula were prepared directly by suspending colonies grown for 3 days on an SDA slopes directly in sterile saline solution. Slopes were flooded with 0.85% saline containing 0.5% Tween 20. Fungal growth was gently probed and the resulting suspension was removed and mixed thoroughly with the use of a vortex mixer. After the settling of the larger particles, suspensions were adjusted by using the Macfarland method and diluted as necessary to correspond to final inoculum concentrations 1.5 x 10^6 CFU/ml (National Committee for clinical laboratory standard, 1998).

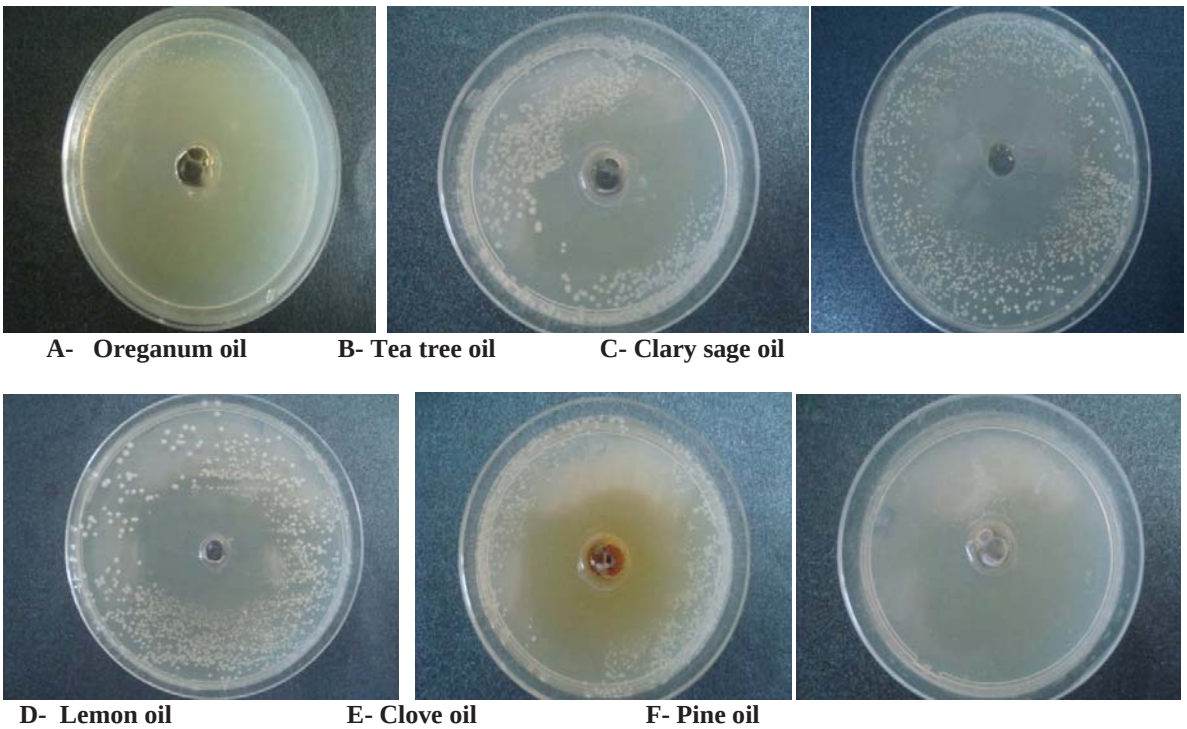


Figure 1: Antifungal activity of some essential and fixed oils on the growth of *Candida abicans* (n5)

Agar-well diffusion susceptibility test

The antifungal activity of essential oils was evaluated against *C. albicans* (n 5), by the agar-well diffusion method. Petri dishes with a diameter of 15 cm were prepared with Saboraud dextrose agar (SDA). The wells (6 mm in diameter) were then cut from the agar by the use of a cork borer and 0.100 mL of essential oil or drugs was delivered to them. The oil was dissolved in tween 80% to obtain the test concentrations of 20 mg/mL. Stock solutions of Nystatin(0.005 mg/ml) was prepared in distilled water and tested as positive controls for *Candida* spp. Each fungal suspension was

inoculated onto the surface of the agar. After incubation, for 3–5 days for *Candida* spp.. at 28°C , all dishes were examined for zones of growth inhibition and the diameters of these zones were measured in millimetres. Each experiment was repeated at least twice.(Brito et al.,2006)

Table 1. Antifungal activity of some essential oils against the growth of different strains of *Candida albicans* spp.

Oil used (25µl/well)	Average inhibition zone (mm)				
	Candida albicans n_1	*Candida albicans* n_2	*Candida albicans* n_3	*Candida albicans* n_4	*Candida albicans* n_5
Pine	47.0^{d}	55.1^{d}	41.2^{d}	45.1^{d}	50.0^{d}
Oreganum	72.2^{f}	74.2^{f}	71.4^{f}	70.2^{f}	75.2^{f}
Clary Sage	50.0^{d}	51.4^{d}	59.0^{d}	55.2^{d}	59.0^{d}
Tea Tree	38.1^{c}	40.1^{c}	35.1^{c}	40.4^{c}	41.3^{c}
Thyme	37.3^{c}	31.0^{c}	32.3^{c}	32.3^{c}	35.2^{c}
Clove	30.1^{c}	22.6^{b}	28.2^{b}	30.2^{c}	28.1^{b}
Rose	13.0^{a}	12.0^{a}	10.0^{a}	10.0^{a}	11.0^{a}
Eucalyptus	23.2^{b}	21.2^{b}	28.2^{b}	17.6^{b}	19.0^{ab}
Lemon grass	21.4^{b}	20.1^{b}	18.4^{ab}	16.4^{b}	14.0^{a}
Lavender	11.0^{a}	10.0^{a}	12.0^{a}	10.0^{a}	10.0^{a}

Means in each column having the same subscript letters are not significantly different at $P \leq 0.05$.

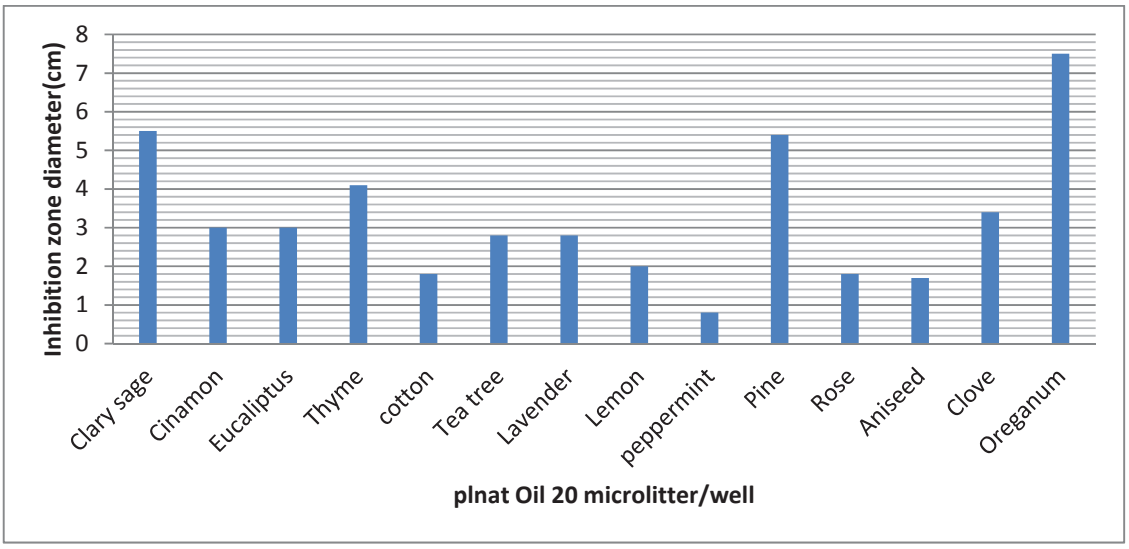


Figure 2: The inhibition zone measurements of some essential and fixed oils against the growth of *Candida albicans*(n5)

Broth microdilution method

The MIC and minimum fungicidal concentration (MFC) for *C. albicans* were determined by the broth microdilution method. The microdilution assay was performed in 96-well microdilution plates. Growth and sterile control wells were included for each isolate tested. The microplates were incubated at 37° C and read visually after 2 days for *Candida* spp . All isolates were run in duplicate and repeated at least twice. The MIC was defined as the lowest oil concentration that caused 80% inhibition of visible fungal growth. The results were read visually, The MFC was determined by subculturing 100 mL of solution from wells without turbidity, on potato dextrose, at 28^0 C. The MFCs were determined as the lowest
concentration resulting in no growth on the subculture after 2 days for *Candida* spp. (Brito et al.,2006)

Table2: Minimal inhibitory concentration (MIC) of selected oils against different strains of *Candida albicans* (ungerminted cells).

Oil used (25µl/ml)	Minimum Inhibitory Concentration (µl/ml)				
	Candida albicans n_1	*Candida albicans* n_2	*Candida albicans* n_3	*Candida albicans* n_4	*Candida albicans* n_5
Pine	1.95^a	1.95^a	1.95^a	1.95^a	7.81^b
Oreganum	0.12^a	0.48^a	0.12^a	0.48^a	0.48^a
Clary Sage	7.81^b	1.95^a	7.812^b	31.25^b	1.95^a
Tea Tree	7.81^b	31.25^b	125.00^c	31.35^b	31.25^c
Thyme	31.25^c	31.25^b	125.00^c	125.00^c	31.25^c
Clove	125.00^d	31.25^b	125.00^c	125.00^c	125.00^d
Rose	500.00^f	500.00^d	500.00^d	500.00^d	500.00^f
Eucalyptus	125.00^d	31.25^b	500.00^d	125.00^c	125.00^d
Lemon grass	125.00^d	125.00^c	500.00^d	500.00^d	500.00^f
Lavender	500.00^f	500.00^d	500.00^d	500.00^d	500.00^f

Means in each column having the same subscript letters are not significantly different

at $P \leq 0.05$.

Time- kill curve procedure

Time- kill studies were carried out against *candida albicans* (n_1, n_2, n_3, n_4 and n_5) germinated and ungerminated cells. Starting inoculums concentration of 1.5×10^5CFU/ml. Based on the data obtained from MIC and MFC for the most effective oils, concentration was chosen that corresponds to 2×MIC. Germinated and ungerminated cells were incubated in 1 ml of PYG broth at 35 °C for 24 hrs in the presence and absence (control) of various chosen oils. Further samples were taken at time intervals (2, 4, 6, 8 and 24hrs) for viable counts which were carried out by serial dilution of samples by 10 fold in sterile distilled water and plating on SDA(p. 24) and the results were estimated according to log values (Hammer et al., 2002).

Table 3. Minimal fungicidal concentration (MFC) of selected oils against different strains of *Candida albicans* (ungerminated cells).

Oil used (25µl/ml)	Minimum Fungicidal Concentration (µl/ml)				
	Candida albicans n_1	*Candida albicans* n_2	*Candida albicans* n_3	*Candida albicans* n_4	*Candida albicans* n_5
Pine	7.81^{b}	31.25^{b}	7.81^{b}	1.95^{a}	7.81^{b}
Oreganum	1.95^{a}	1.95^{a}	0.48^{a}	0.48^{a}	0.48^{a}
Clary Sage	31.25^{c}	1.95^{a}	1.95^{a}	31.25^{b}	1.95^{a}
Tea Tree	31.25^{c}	125.00^{c}	31.25^{c}	125.00^{c}	31.25^{c}
Thyme	31.25^{c}	125.00^{c}	125.00^{d}	125.00^{c}	31.25^{c}
Clove	125.00^{d}	125.00^{c}	125.00^{d}	500.00^{f}	125.00^{d}
Rose	500.00^{f}	500.00^{d}	500.00^{f}	500.00^{f}	500.00^{f}
Eucalyptus	125.00^{d}	125.00^{c}	500.00^{f}	500.00^{f}	500.00^{f}
Lemon grass	500.00^{f}	500.00^{d}	500.00^{f}	500.00^{f}	500.00^{f}
Lavender	500.00^{f}	500.00^{d}	500.00^{f}	500.00^{f}	500.00^{f}

Means in each column having the same subscript letters are not significantly different

at $P \leq 0.05$.

Transmission Electron microscope (TEM)

On the basis of MIC, MFC values and time-kill curve data. *Candida albicans* (n_1) germinated and ungerminated cells were treated with pine oil (7.81µl/ml), clary sage oil (31.25µl/ml), oregano oil (1.95µl/ml) and tea tree oil (31.25µl/ml) for 24 hrs (MFC endpoint). Freshly taken samples were fixed using a universal electron microscope fixative as described by McDowell & Trump (1967). Series dehydration steps were followed using ethyl alcohol and propylene oxide. The samples was then embedded in labeled beam capsules and polymerized. Thin sections of cells exposed to oils were cut using LKB 2209-180 ultramicrotome and stained with a saturated solution of urinyl acetate for half hour and lead acetate for 2 min (McDowell &Trump, 1967). The procedure was applied to control cells not exposed to oils and to oil-exposed cells. Electron Micrographs were taken using a Transmission electron microscope (JEM-100 CX Joel), at the Electron Microscope Unit, Faculty of Science, Alexandria University.

Statistical analysis

Statistical analysis were performed using SPSS 17-0 soft ware for windows (Statistical Product and Services Solutions,Inc,Chicago,IL,USA).A *p* of 0.05 was set as the significant threshold for all statistical analysis.

Results & Discussion:

Screening for the Antifungal Activity of the Essential (Volatile) and Fixed oils
Measurments of in vitro activity of plant essential oil against *Candida* spp.

Data listed in Table 1 pesents the antifungal activity of 18 essential and fixed oils on the growth of *Candida albicans* spp (n1-n5). Oregano oil was found to be the most effective and significant antifungal agent on the growth of *Candida albicans* (n_1, n_2, n_3, n_4 &n_5) as compared to the other oils tested showing an average inhibition zone of 72 mm followed by several oils in the order of clary sage oil (55 mm) > pine oil (47 mm) > tea tree oil (39 mm) > thyme oil (33 mm) > clove oil (27 mm) > eucalyptus oil (21 mm) > lemon grass oil (18 mm). Whereas rose and lavender oil (11 mm) showed the least and the same antifungal effect $P \leq 0.05$. Otherwise all the *Candida albicans* strains under test were found to be resistant to almond, sesame, fennel, linseed, castor, cactus, cumin and black seed oils. Devkatte et al. (2005) revealed that oregano, tea tree, thyme, clary sage, clove, eucalyptus, lemongrass, rose and lavender exhibited a broad spectrum of antifungal activity with an average inhibition zones of (47±3, 36±4, 35±1.2, 34.3±1.2, 23±1, 9±1, 27, 10 mm respectively) against *Candida albicans* strains SRTCC I, SRTCC II and SRTCC III (Devkatte et al., 2005; Amit & Shailendra, 2006; Rusenova, 2009 and Agarwal et al., 2010).
Oregano oil exhibited a significant variation of MIC values (7.5×10-3-0.03µl/ml) against all *Candida albicans* strains under investigation, followed by pine oil (0.12-0.48µl/ml), clary sage (0.48-1.95µl/ml) and tea tree oil (1.95-7.81µl/ml). Whereas thyme and clove oil showed moderate antifungal property with MIC values between 7.81 and 31.25 µl/ml, eucalyptus and lemon grass oil showed MIC values between 31.25 and 125 µl/ml, while lavender and rose oil showed the least MIC value between 125 and 500 µl/ml against *Candida albicans* strains under investigation $P \leq 0.05$. On the basis of the previous data, the most promising oils that proved to have the most powerful inhibitory effect on the growth of Candida albicans strains under investigation were selected for the detection of the minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the selected essential oils (oregano, lavender, clove, rose, clary sage, eucalyptus, lemongrass, thyme, pine and tea tree oils) that proved to be strong and moderate inhibitors for the growth of *Candida albicans* under test with MIC values ≤ 500µl/ml. However, germinated *Candida albicans* reveal efficient MIC & MFC more than ungerminated. Data of the The present work is in agreement with the study done by Rosenova et al. (2009) which found that oregano oil was the most effective antifungal agent against *Candida albicans* isolates at a low concentration of 0.06%(v/v) followed by thyme and clary sage of MIC value equal to 0.5 %(v/v); tea tree oil 1%(v/v) and clove oil 0.25%(v/v). Moreover, the present work is not in conformity with Agarwal et al. (2010) who found that eucalyptus oil was the most effective oil against *Candida albicans* isolates with MIC value equal to 0.05%(v/v) followed by clove 0.33%(v/v) and tea tree oil 0.33%(v/v) (table 2&3). Fungicidal activity of the tested oils was ranging from 7.5×10^{-3}-125 µl/ml for germinated and 0.48-500 µl/ml for ungerminated cells of *Candida albicans* strains (n_1, n_2, n_3, n_4 &n_5) under test. The best MFC value was observed for clary sage, pine, oregano and tea tree oil with germinated and ungerminated cells(table 3) . The antimicrobial activities of these plant oils may possibly be due to the presence of carvacrol and p-Cymene (Manohar et al., 2001 ; Baser, 2002 and Curillo-Munoz et al.(2006). According to Rusenova (2009) oreganum oil was effective against *P. vulgaris, B. licheniformis, M. pachydermatis, S. aureus* and *C. albicans* isolates.
Time- kill assay demonstrated that *Candida albicans* (n5) was highly susceptible to tested oils. showed that the number of colonies for germinated cells was significantly reduced after 12 hrs of incubation and the total fungicidal effect was observed within 24 hrs of contact for all tested oils. The oils under test exerted a rapid fungicidal effect under shaken conditions.While the number of colonies for ungerminated cells was significantly reduced after 24 hrs of incubation and the total fungicidal effect was observed within 48 hrs of contact for all the tested oils. The oils under test, showed a slower fungicidal effect under static conditions (48 hrs) than under shaken conditions (24 hrs)(fig.3&4).

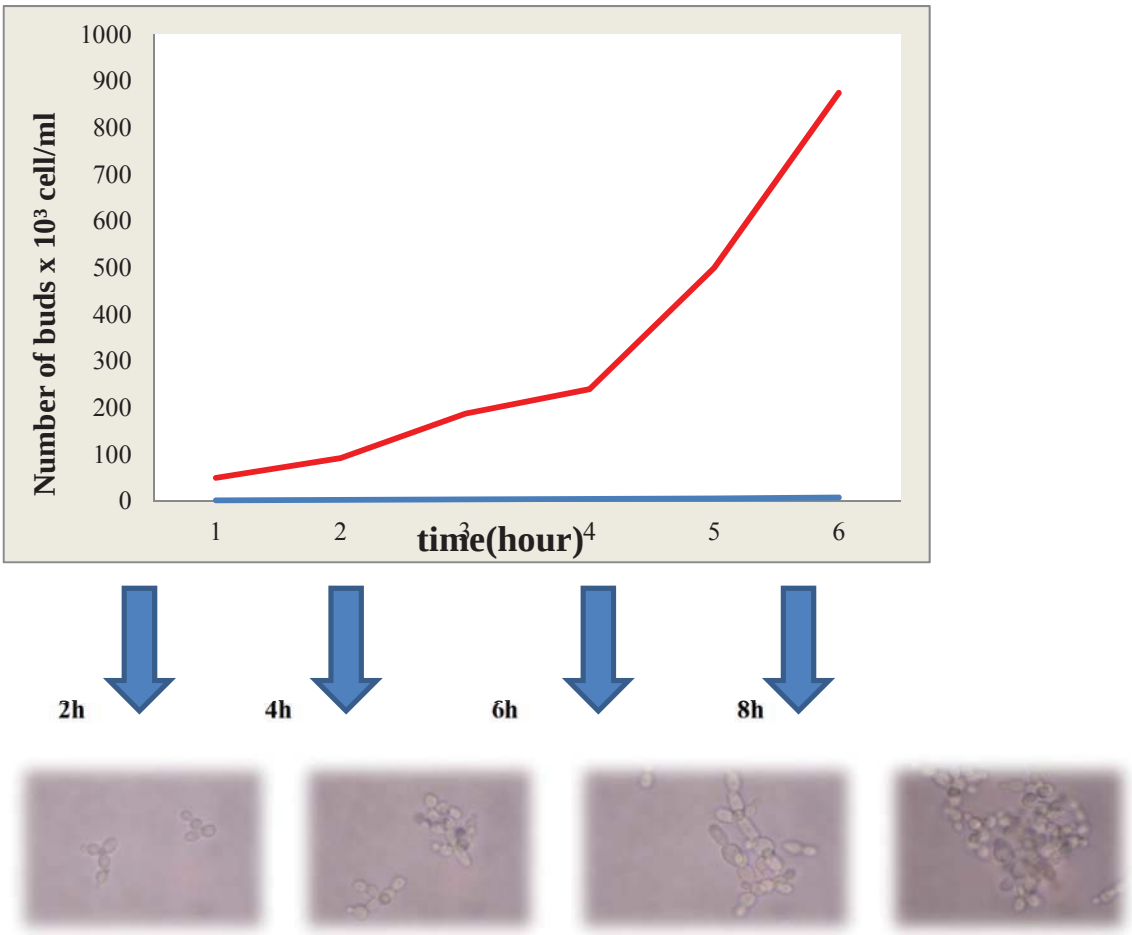


Figure 3: Budding growth curve of *Candida albicans* (n5)growing on peptone yeast glucose (PYG) medium

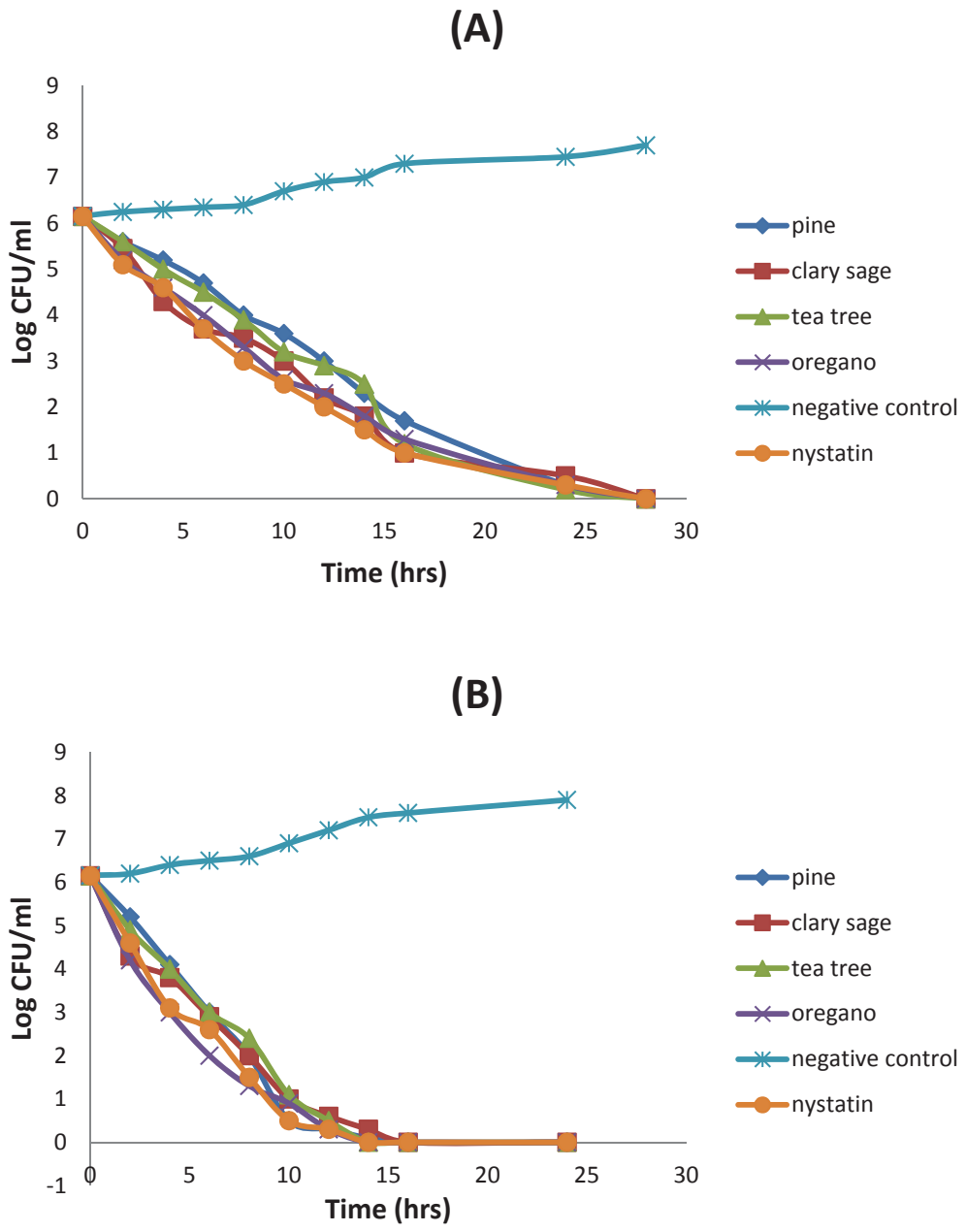
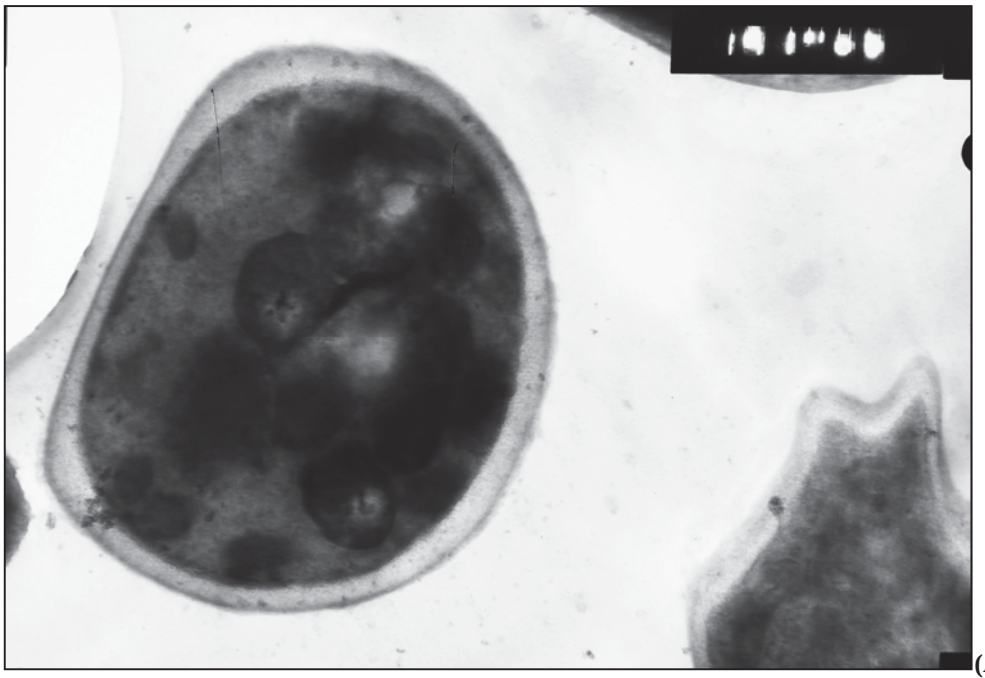
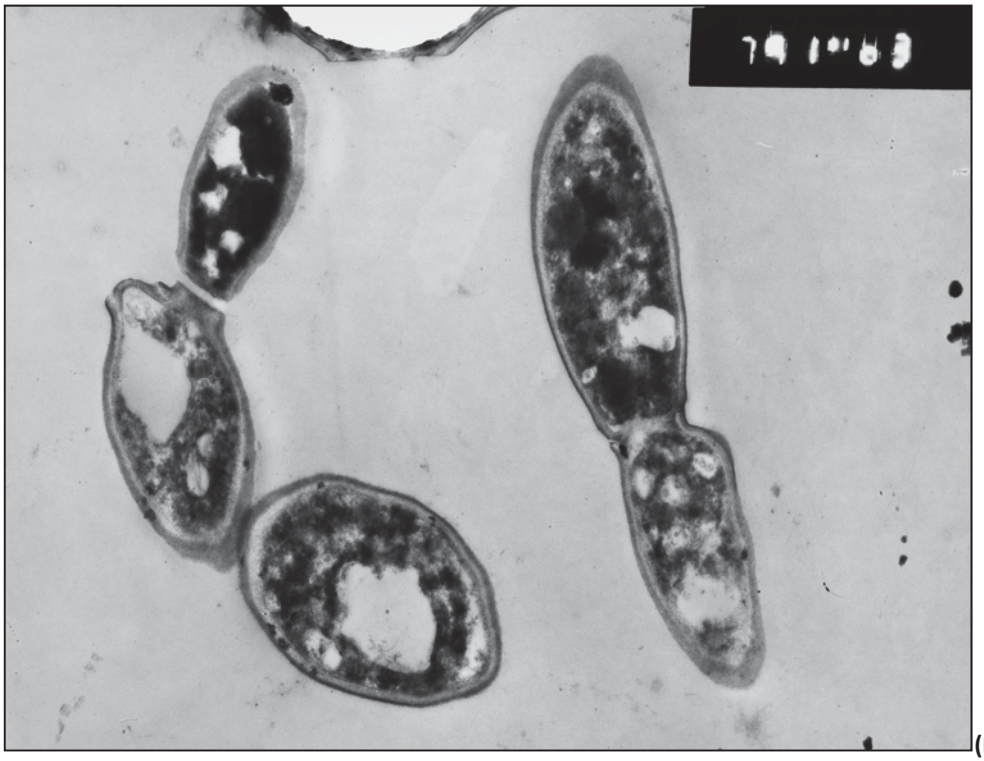


Figure 4: Time-Kill curve of the selected oils against *Candida albicans*(n_5)germinated cells under static (A) and shaken (B) conditions

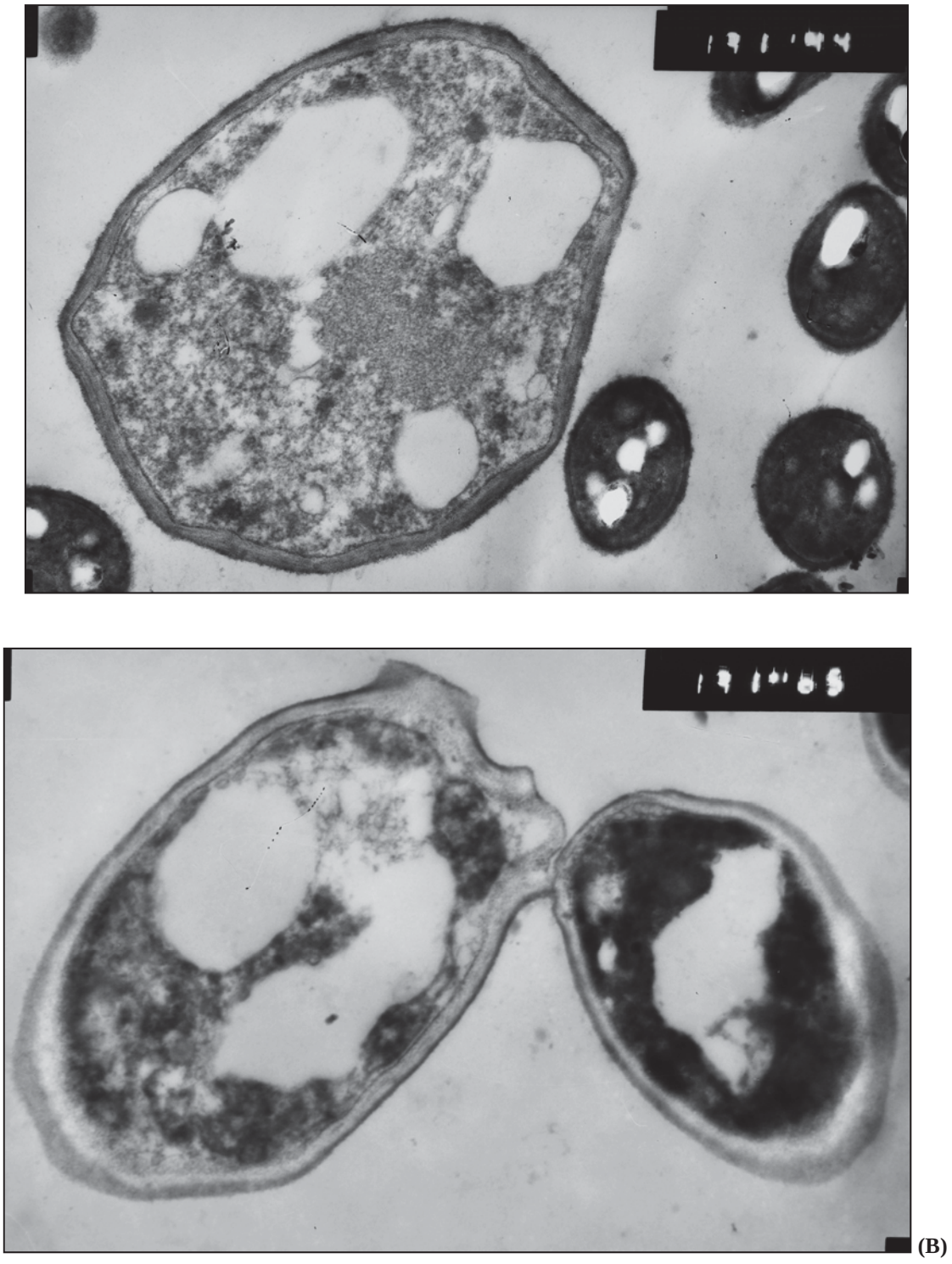


(A)



(B

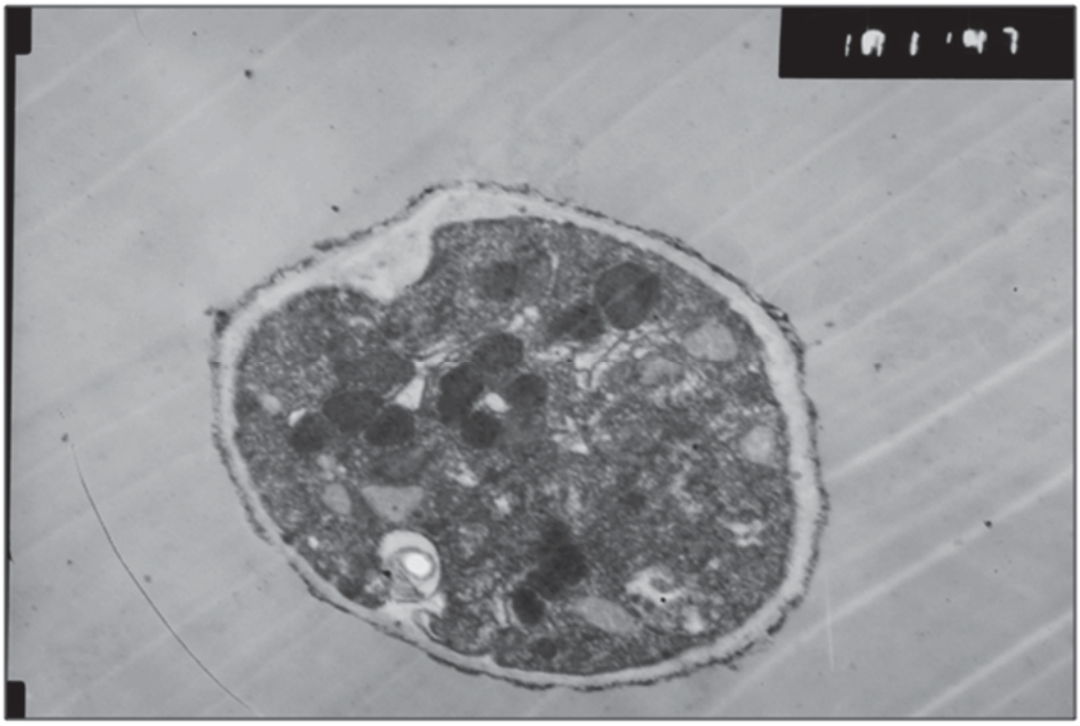
Figure 5. Transmission electron micrographs of *Candida albicans* (n_5) ungerminated (A) and germinated control (B) {*cm*: cytoplasmic membrane, *cw*: cell wall and *v*: vacuoles}



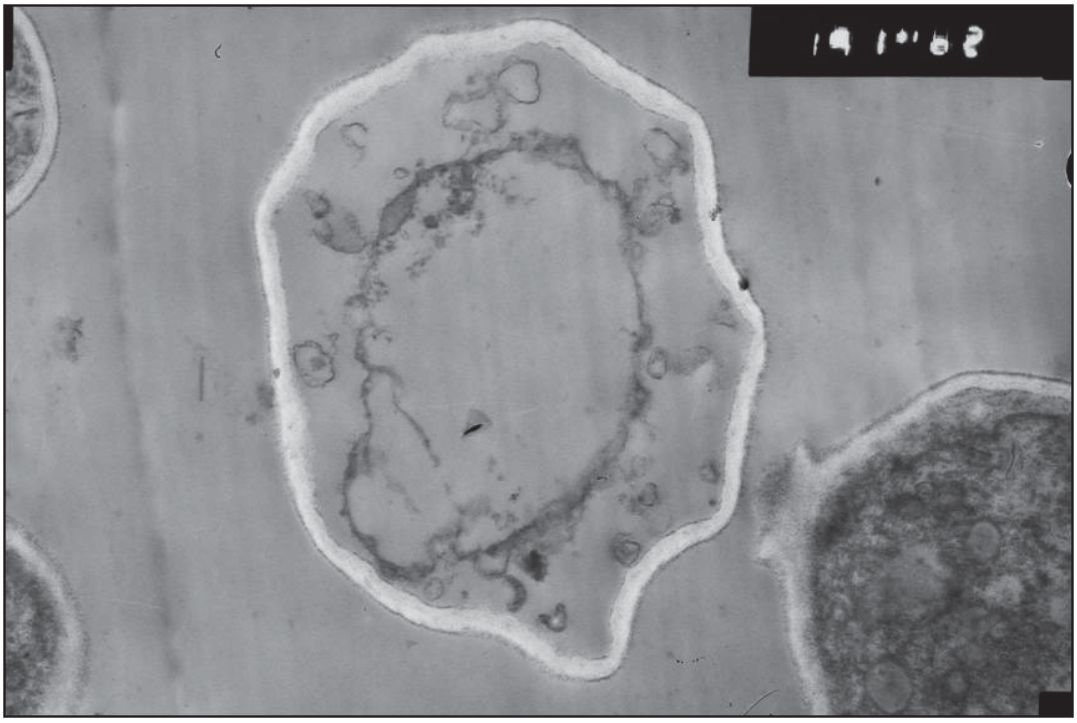
(A)

(B)

Figure 6. Transmission electron micrographs of *Candida albicans* (n_5) treated with pine oil ungerminated cells (A) and germinated cells (B)

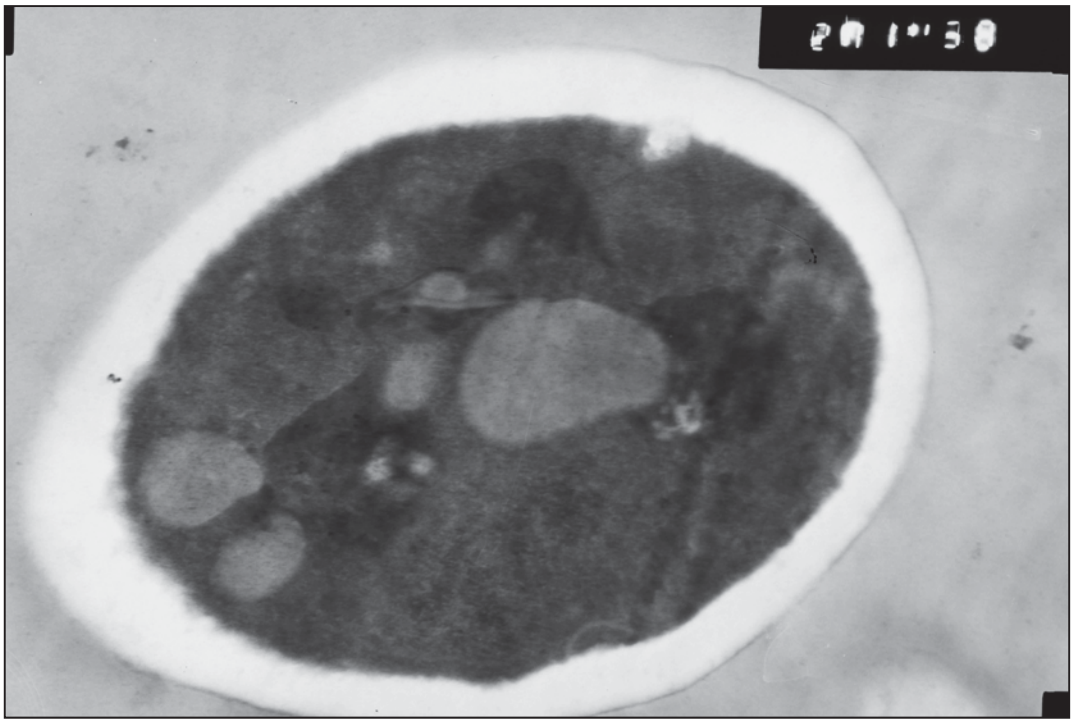


(A)

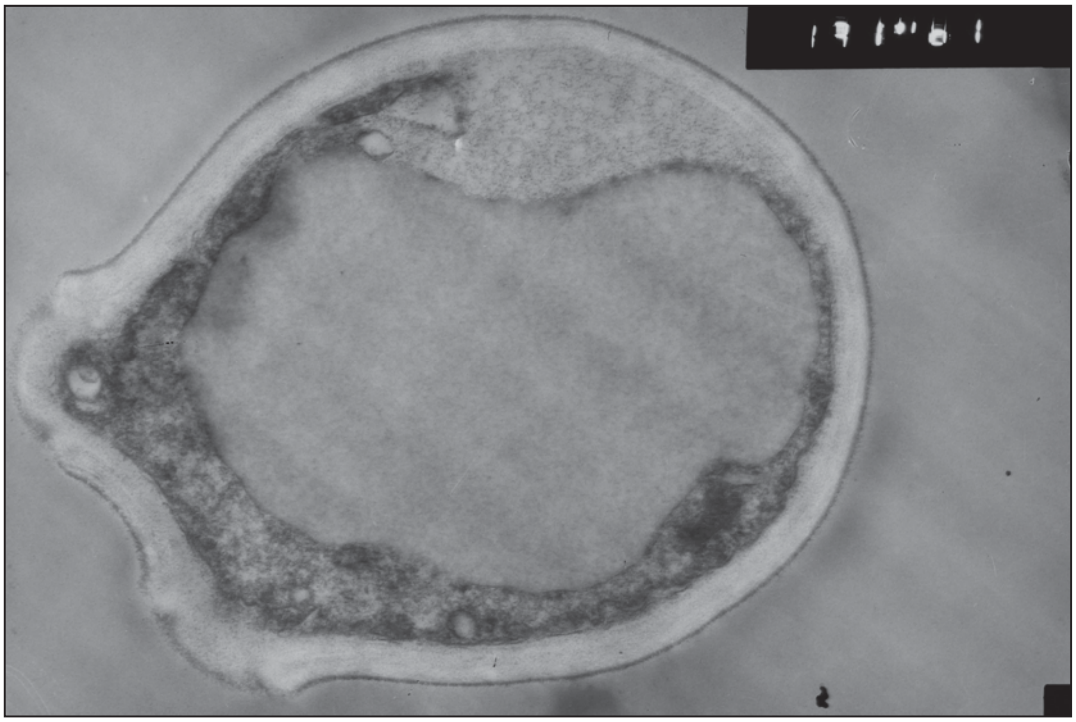


(B)

Figure 7. Transmission electron micrographs of *Candida albicans* (n_5) treated with tea tree oil ungerminated cells (A) and germinated cells (B)

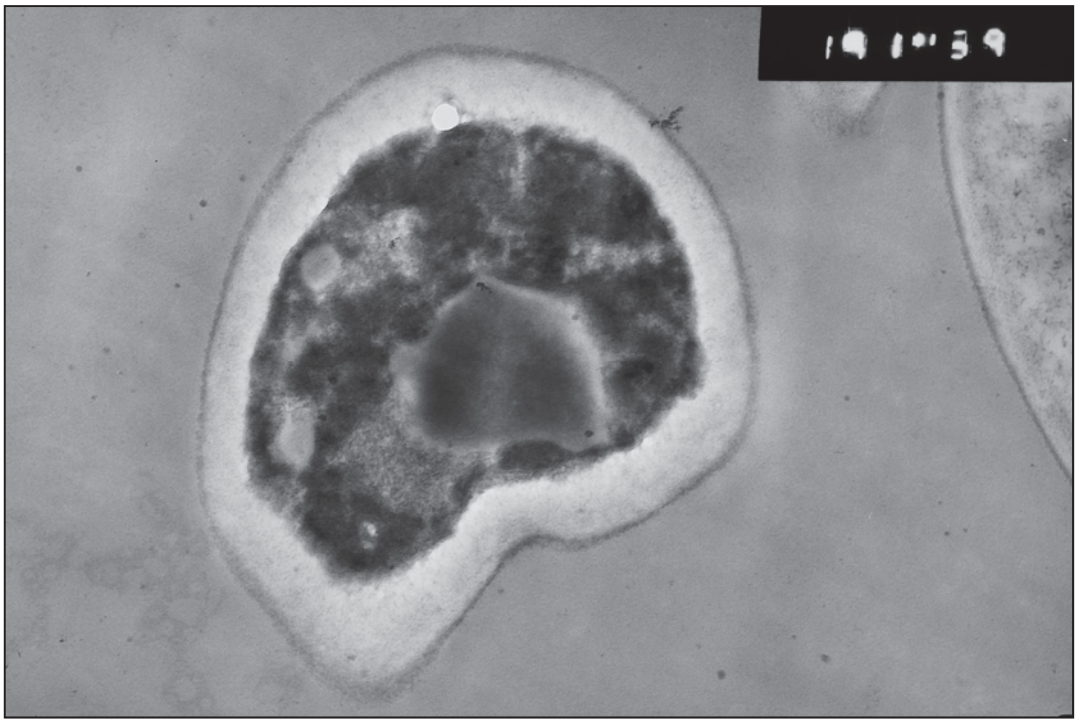


(A)

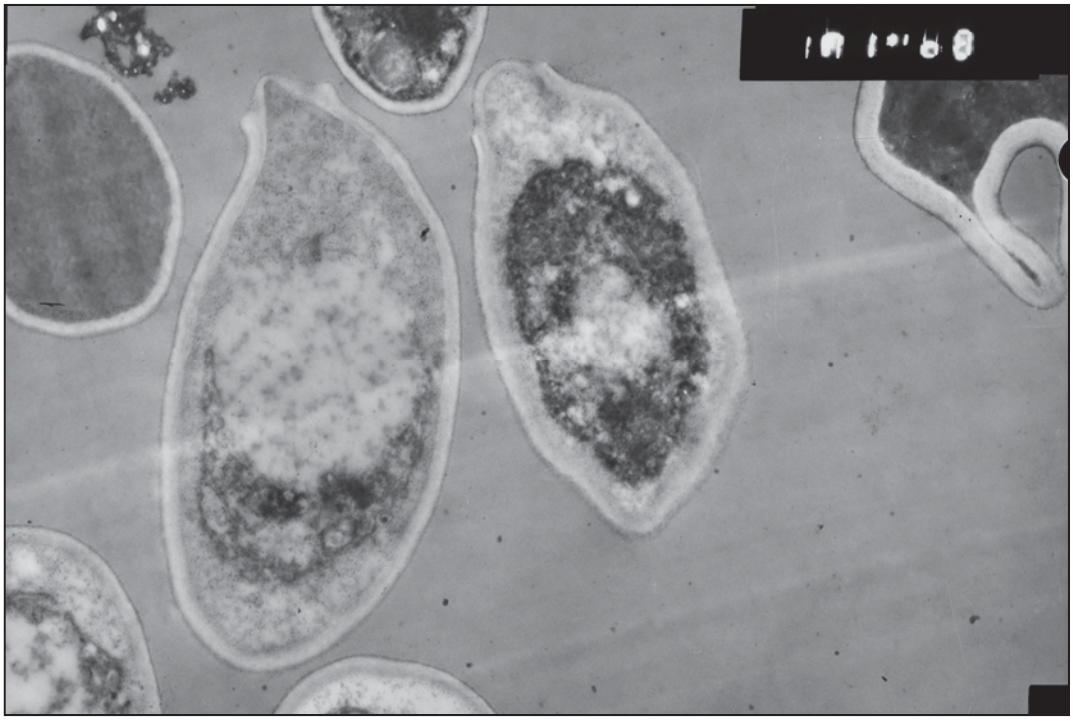


(B)

Figure 8. Transmission electron micrographs of *Candida albicans* (n_5) treated with clary sage oil ungerminated cells (A)and germinated cells (B)



(A)



(B)

Figure 9. Transmission electron micrographs of *Candida albicans* (n_5) treated with oregano oil ungerminated cells (A)and germinated cells (B).

The oil toxicity against *Candida albicans* cell was tested by transmission electron microscopy. The untreated cells (Control) showed a typical morphology of *Candida* cells with a uniform central density, with intact intracellular structures and envelope and intact cell wall for germinated cells and ungerminated cells.
The cytoplasm of ungerminated cells treated with pine oil did not appear to be homogeneous. Whereas, germinated cells underwent pronounced morphological alterations and loss of integrity of the cell wall. Tea tree oil caused notable alterations in the ultrastructure of the germinated and ungerminated cells as compared to control. Increased granulation of the cytoplasm and diminished cell membrane clarity noticed in germinated than ungerminated cells in comparison with the control.Moreover, ungerminated cells treated with clary sage oil, underwent a partial destruction in the internal membranes, with complete destruction of the cell wall and cell lysis in germinated cells. However, ungerminated cell treated with oregano oil showed very dense with vesicles dispositioned within the cell. The cells exhibited notable alterations in the cell membrane and the cell wall forming structural disorganization within the cell cytoplasm(fig. 5-9).

References

Agarwal, V.; Lal, P. and Pruthi, V. (2010). Effect of Plant Oils on *Candida albicans.* J Microbiol Immunol Infect., 43:447-451

Amit, R. and Shailendra, S. (2006). Ethnomedicinal approach in biological and chemical investigation of phytochemicals as antimicrobials. Latest Rev., 4:103-111.

Baser, K.H.C. (2002). Aromatic biodiversity among the flowering plant taxa of Turkey. Pure Appl Chem., 74: 527-545.

Brito EHS, Fontenelle ROS, Brilhante RSN et al. (2006) Phenotypic characterization and in vitro antifungal sensitivity of Candida spp. And M. pachydermatis strains from dogs. Vet J; doi: 10.1016/ j.trjl.2006.05.021.

Chaieb K, Zamantar T, Ksouri R, et al.(2007)Antioxidant properties of the essential oil of *Eugenia caryophyllata* and its antifungal activity against a large number of clinical *Candida* species. *Mycoses*.; 50: 403-6.

Çitak S, Ozcelik B, Cesurm S, Abbasoglu U. (2005) *In vitro* susceptibility of *Candida* species isolated from blood culture to some antifungal agents. Jpn J Infect *Dis*.; 58: 44-6.

Curillo-Munoz AJ, Quindos G, Ruesga M, et al.(2006) *In vitro* anti fungal susceptibility testing of filamentous fungi with sensitive Yeast OneTm. Mycoses.
49: 293-7.

Deak,T(1986). Identification of food borne yeast . food post.,50:243-264

Devkatte AN, Zore GB, Karuppayil SM. *(* 2005) Potential of plant oil as inhibitors of *Candida albicans* growth. FEMS Yeast Research5: 867-73.

Hammer, K.A.; Carson, C.F. and Riley, T.V. (2002). In vitro activity of Melaleuca alternifolia (tea tree) oil against dermatophytes other filamentous fungi. J Antimicrobial Chemother, 50: 195–9.

Mahdavi Omran S, Esmailzadeh S, Rahmani Z (2009). Comparison of anticandida activity of thyme, pennyroyal and lemon essential oils versus antifungal drugs against *Candida* species. *Jundishapur J Microbiol*. 2: 53-60.

Manohar, V.; Ingram, C.; Gray, J.; Talpur, N.A.; Echard, B.W.; Bagchi, D. and Preuss, H.G. (2001). Antifungal activities of origanum oil against *Candida albicans*. Mol. Cellular Biochem.. 228: 111-117.

McDowell, E.M. and Trump, B.F. (1967). Histoligic fuxative suitable for diagnostic light and electron microscopy. Arch pathol lab., 10:405-413

Motsei ML, Lindsey KL, van Staden J, Jager AK.(2003) Screening of traditionally used South African plants for antifungal activity against *Candida albicans*. Ethnopharmacol. 86(2-3): 235-41

National Committee for Clinical Laboratory Standards (1998). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Conidium-Forming Filamentous Fungi: Proposed Standard M38-P. NCCLS, Wayne, PA, USA.

Rusenuva, N. and Parvanov, P. (2009). Antimicrobial activities of twelve essential oils against microorganisms of veterinary importance. J.Trakia. Sci., 7:37-43