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Full Length Research Article

Anti-fungal activity of plant oils against oral clinical isolates of *Candida albicans* in Lebanese community

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Abstract

The objective of this is to investigate the antifungal activity of 10 available essential oils against *Candida albicans in vitro* and to compare the antifungal activity between each material. Oils extracted from different medicinal plants (volatile and fixed) were screened for their activity against oral pathogen *C. albicans* by agar well diffusion method. The inhibitory effect of oils against *C. albicans* was done by agar diffusion method, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFCs) using the broth micro dilution and agar dilution methods were evaluated. The results showed that the maximum antifungal activity was obtained by pine, tea tree, *oreganum*, thyme and clary sage oil as compared to nystatin as a control. While clove, eucalyptus, cinnamon, lemon and lavender oils exhibited moderate inhibitory activity. Cotton, aniseed and rose oils demonstrated low activity as compared to control. Mustard, linseed, peppermint, sesame, fennel, caraway, cumin, cactus, castor, blackseed and almond oils have not shown any antifungal activity. More over Time-Kill assay have showed fungicidal activity against *C. albicans* after 6 hours of treatment with the promising oils. These results revealed that the plant oils under investigation can be used to cure fungal infections and plant oils may have pharmaceutical roles.

Key words : Essential oils, *Candida albicans*, anti-fungal activity, *in-vitro* studies.

Introduction

Many essential oils have been advocated for use in complementary medicine against 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).

C. 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**.** *C. 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, Mahdavi et al., 2009)

In the last two decades, some research 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 remedies for headaches, arthritis, 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

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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 essential 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 *C. 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 *C. albicans* using the simplified identification method SIM key proposed by Deak (1986). The yeast strains used in the present study 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.

Essential and fixed oils

Some essential oils used in the present work include: Mustard (Brassica junea), Lavender (*Lavandula officinalis*), Peppermint (Mentha piperita), Anise (Pimpinella anisum), Cloves *(Eugenia caryophyllata*), Fennel (*Foeniculum vulgare)*, Olibanum (Boswwellia sacra), Caraway (Carum carvi), Cumin (Cumin cyminum), Pine (*Pinus*), Rose (*Origanum vulgare*), and Lemon (*Citrus*) oils. The fixed oils used were Almond (*Prunus amygdalus dulcis)*, Castor (Ricinus communis), Sesame (*Prunus amygdalus dulcis*) and Black seed (*Nigella sativa*) oils were obtained from Chahal Brothers North Lebanon.

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).

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., 2007).

Broth microdilution method

The minimum inhibitory (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^o 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° 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., 2007).

Time- kill curve procedure

Time-kill studies were carried out against *C. albicans* (n_1, n_2, n_3, n_4 and n_5) germinated and ungerminated cells. Starting inoculuma 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^o C for 24 h in the presence and absence (control) of various chosen oils. Further samples were taken at time intervals (2, 4, 6, 8 and 24h) for viable counts which were carried

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 superscript letters are not significantly different at $P \leq 0.05$

out by serial dilution of samples by 10 fold in sterile distilled water and plating on SDA and the results were estimated according to log values (Hammer et al., 2002).

Transmission Electron microscope (TEM)

On the basis of MIC, MFC values and time-kill curve data, *C. 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 and 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 and 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 and Discussion

Data listed in Table 1 and Figure 1 present antifungal activity of 18 essential and fixed oils on the growth of *C. albicans* spp (n_1-n_5). Oregano oil was found to be the most effective and significant antifungal agent on the growth of *C. albicans* (n_1, n_2, n_3, n_4 and 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 *C. 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

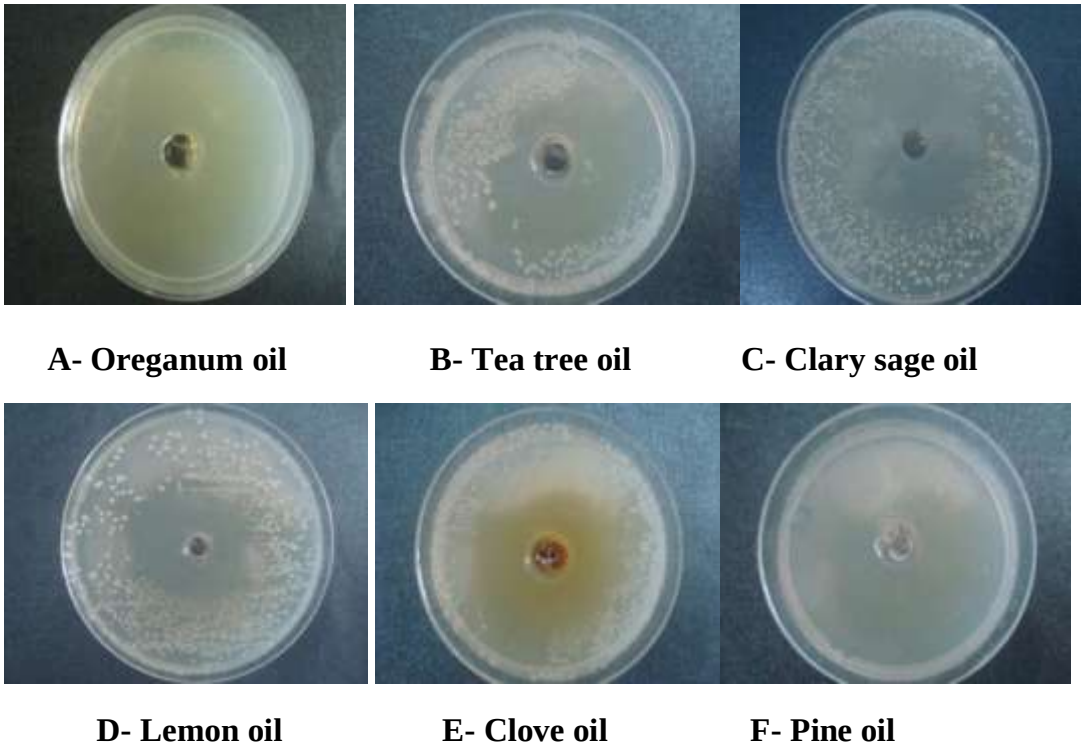


Figure 1: Antifungal activity of some essential and fixed oils on the growth of *Candida albicans* (n_5)

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 *C. albicans* strains SRTCC I, SRTCC II and SRTCC III (Devkatte et al., 2005; Amit and Shailendra, 2006; Rusenuva and Parvanov (2009) and Agarwal et al., 2010 Palá-Paúl et al., 2012). Oregano oil exhibited a significant variation of MIC values (7.5×10^{-3}-0.03µL/mL) against all *C. 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 *C. 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 *C. 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 *C. albicans* under test with MIC values ≤ 500µL/mL. However, germinated *C. albicans* reveal efficient MIC and MFC more than ungerminated. Data of the present work were in agreement with the study done by Rusenuva and Parvanov (2009) which found that oregano oil was the most effective antifungal agent against *C. 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 *C. 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) (Tables 2 and 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 *C. albicans* strains (n_1, n_2, n_3, n_4 and n_5) under test. The best MFC value was observed for clary sage, pine, oregano and tea tree oils 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; Curillo-Munoz et al., 2006; Violante et al., 2012). According to Rusenuva and Parvanov (2009) oreganum oil was effective against *Proteus vulgaris, Bacillus licheniformis, Malassezia pachydermatis, Staphylococcus aureus* and *C. albicans* isolates.

Time- kill assay demonstrated that *C. albicans* (n_5) (Figure 2) was highly susceptible to tested oils. The present study showed that the number of colonies for germinated cells was significantly reduced after 12 hrs of

Table 2. Minimal inhibitory concentration (MIC) of selected oils against different strains of Candida albicans (ungerminted cells)

Oil used (25µl/well)	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 superscript letters are not significantly different at $P \leq 0.05$

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

Oil used (25µl/well)	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	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 superscript letters are not significantly different at $P \leq 0.05$

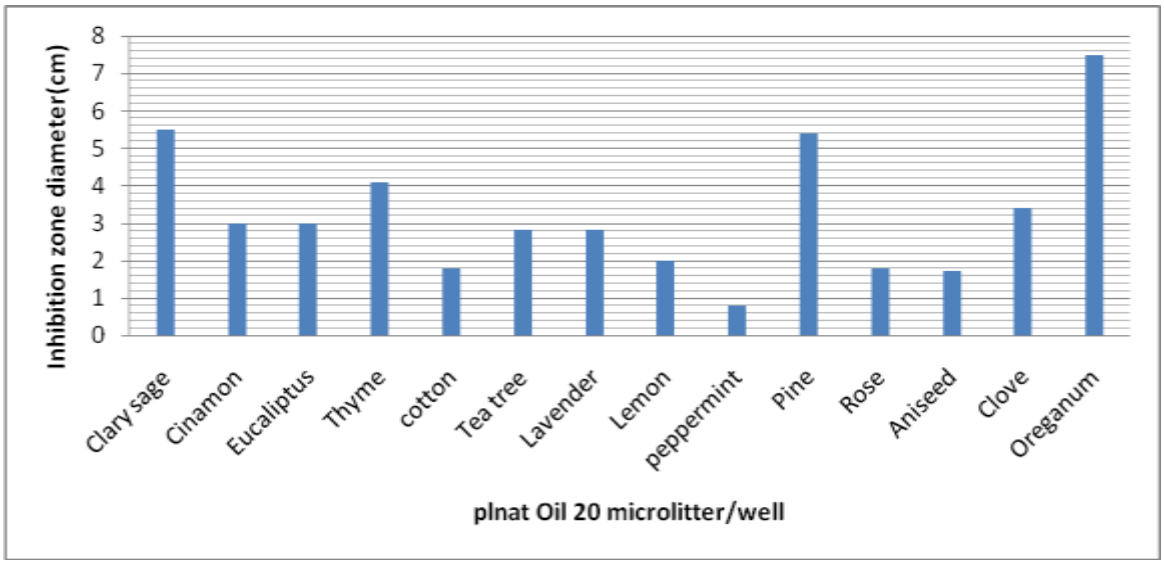


Figure 2: The inhibition zone measurements of some essential and fixed oils against the growth of *Candida albicans*(n_5)

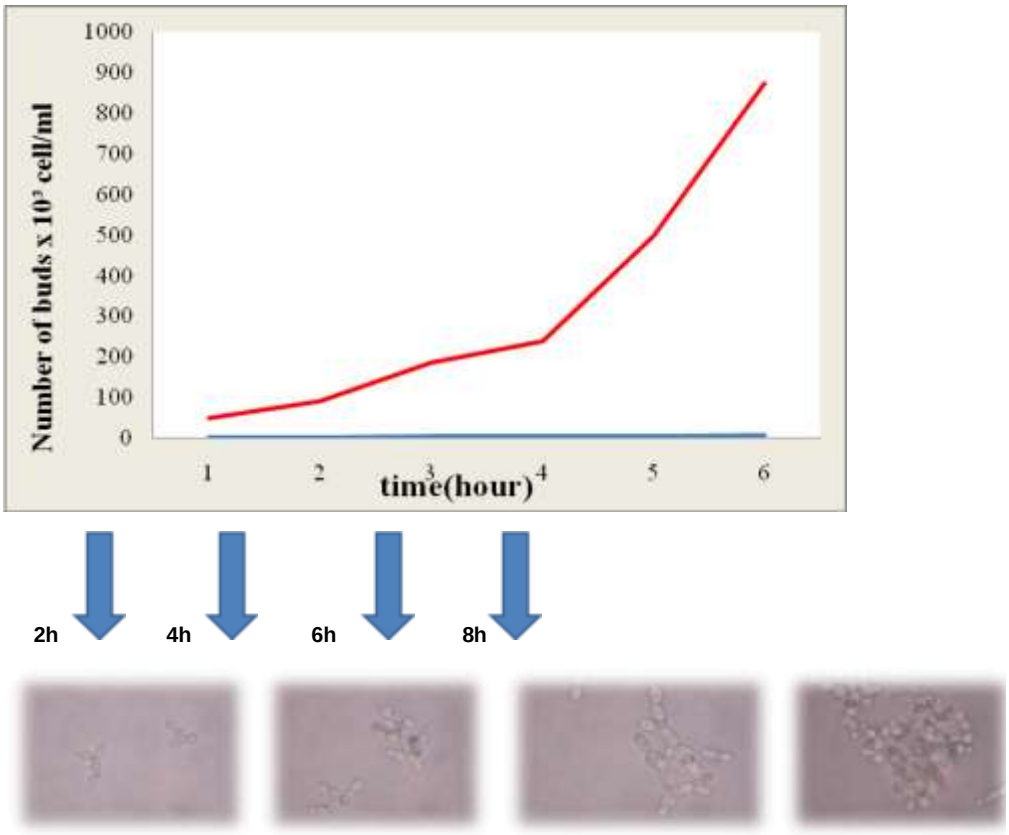


Figure 3: Budding growth curve of *Candida albicans* (n5)growing on peptone yeast glucose (PYG) medium

incubation and the total fungicidal effect was observed within 24 h. 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 h) than under shaken conditions (24 h) (Figure 3 and 4).

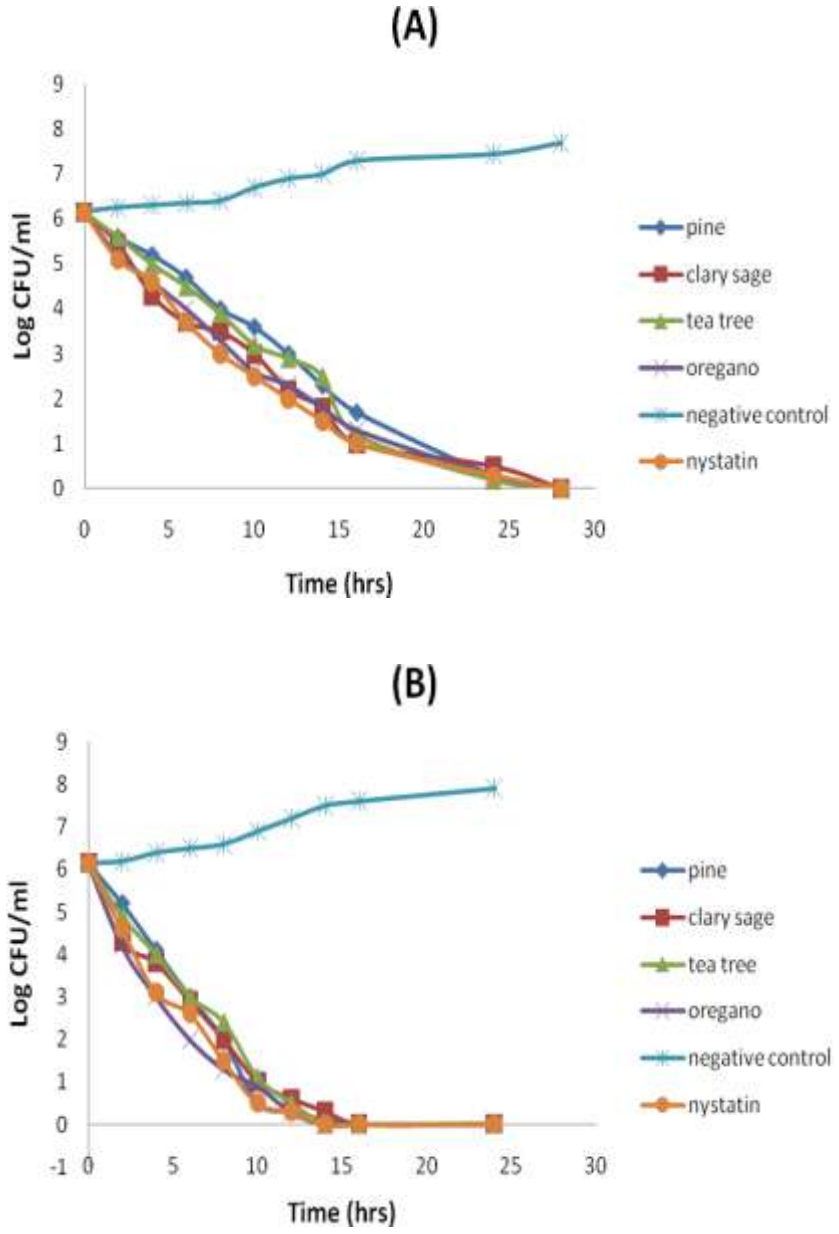


Figure 4: Time-Kill curve of the selected oils against *Candida albicans*(n_5) germinated cells under static **(A)** and shaken **(B)** conditions

The oil toxicity against *C. 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 ultra structure 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 (Figures 5-9)

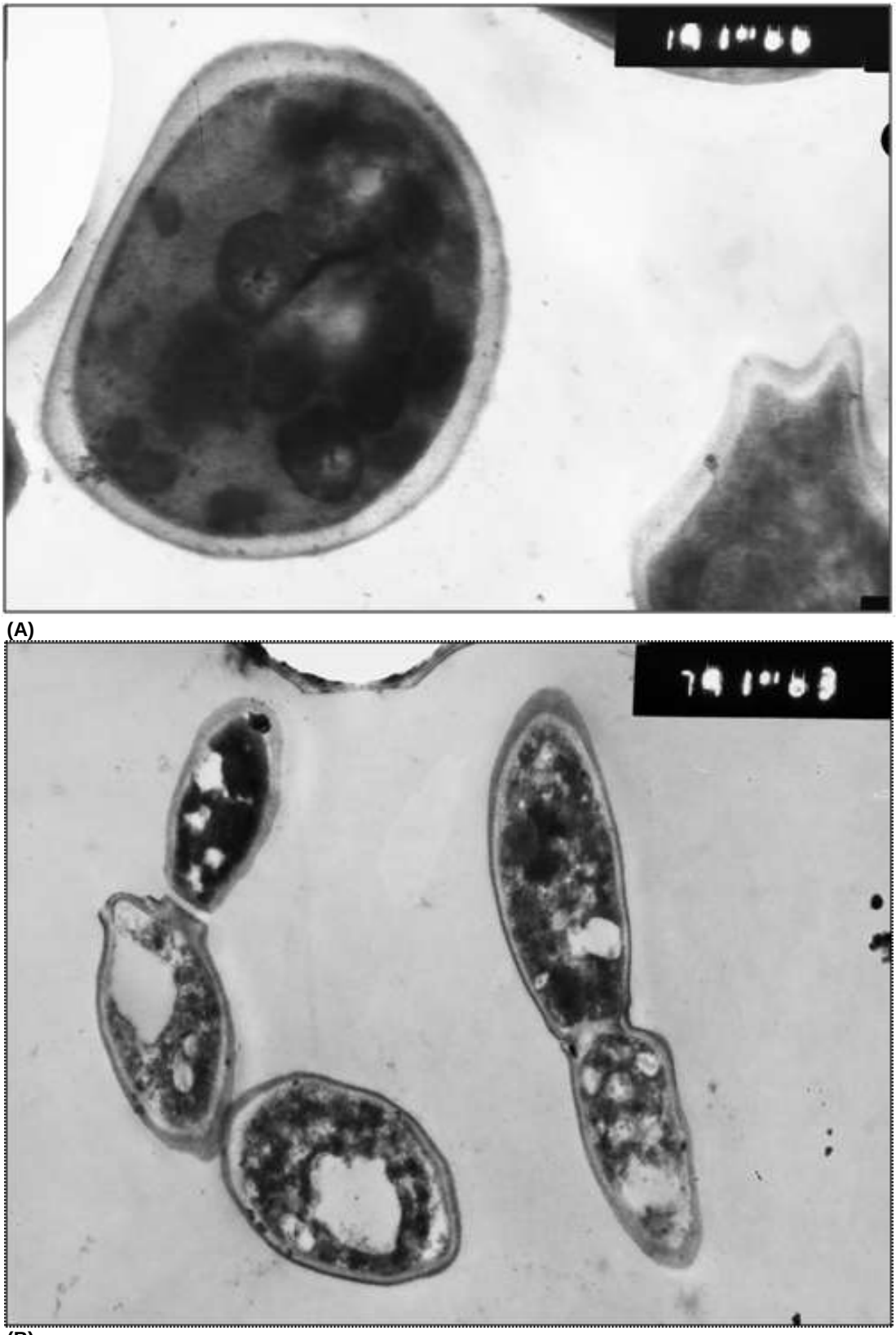


Figure 5. Transmission electron micrographs of *Candida albicans* (n_5) ungerminated (A) and germinated control (B) {*cm*: cytoplasmic membrane, *cw*: cell wall and *v*: vacuoles}

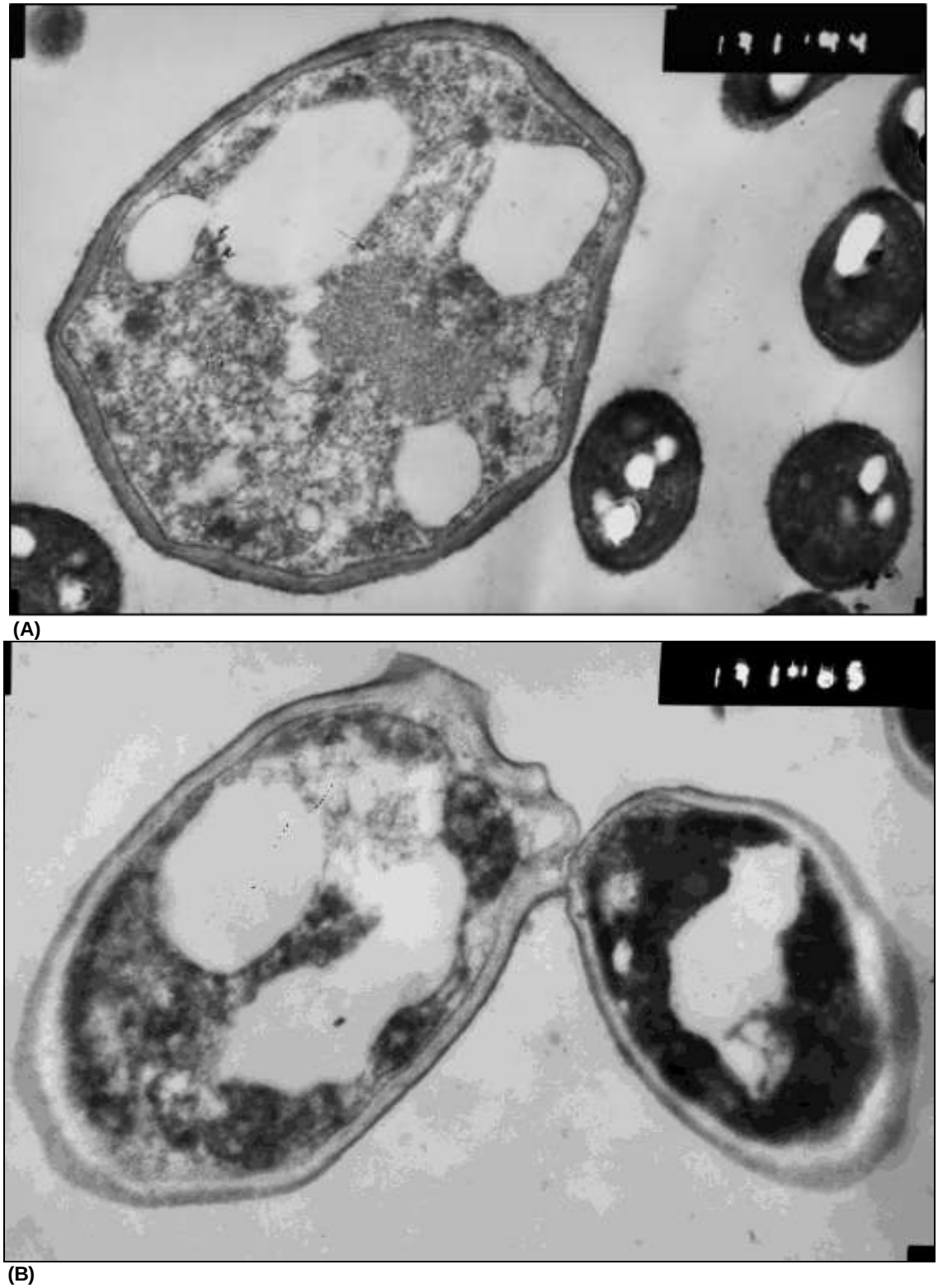
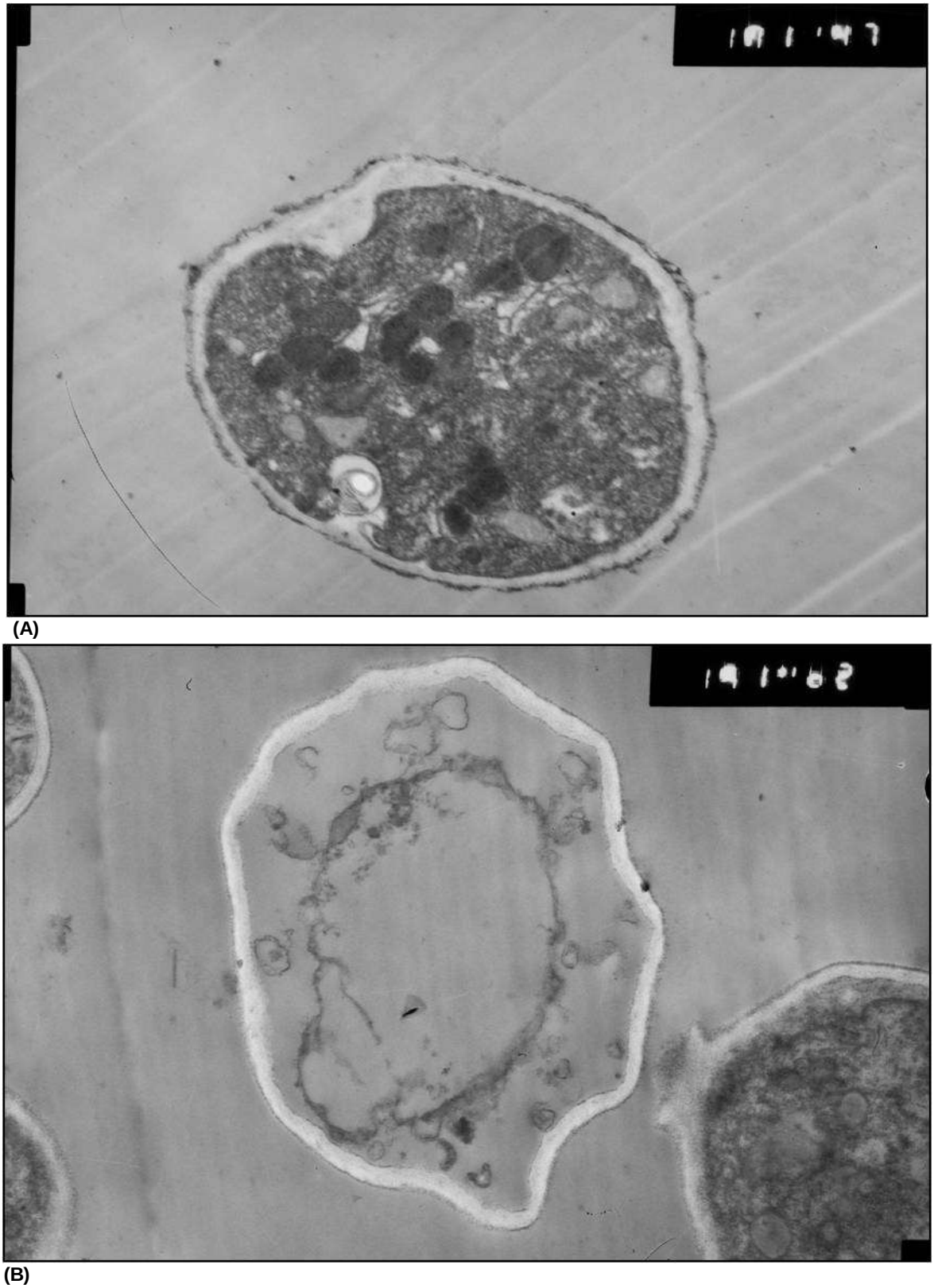


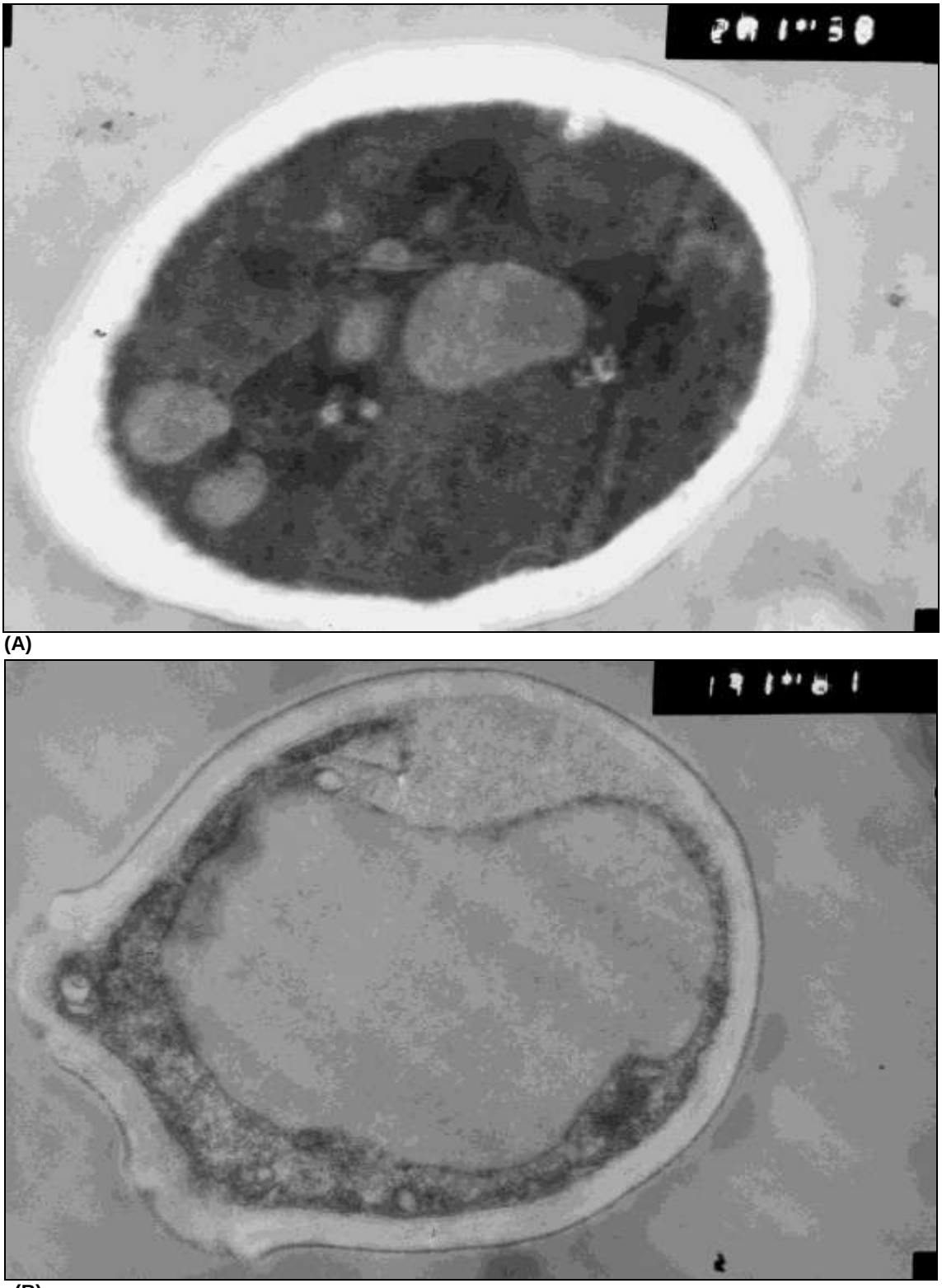
Figure 6. Transmission electron micrographs of *Candida albicans* (n_5) treated with pine oil ungerminated cells (A) and germinated cells (B)



(A)

(B)

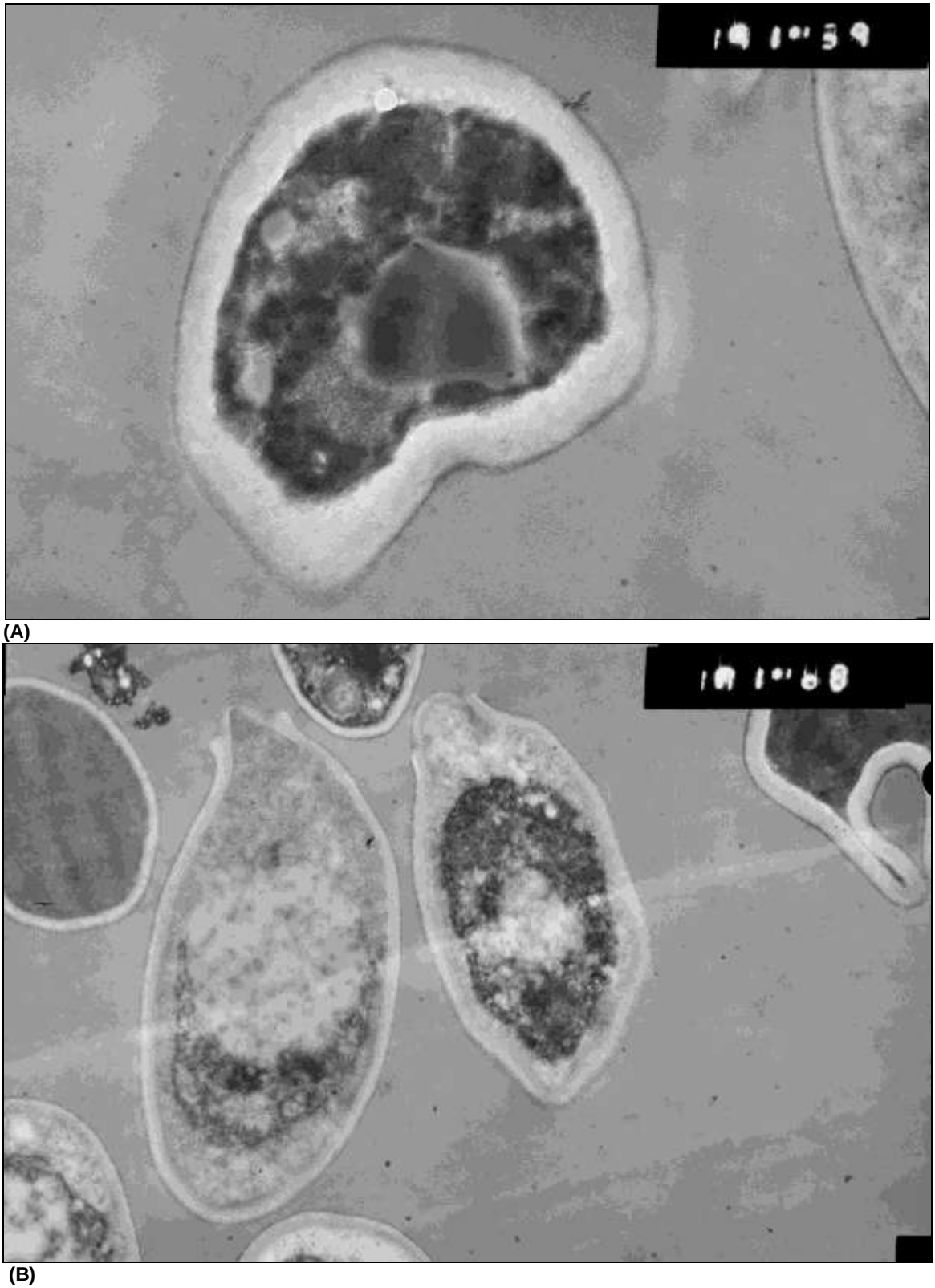
Figure 7. Transmission electron micrographs of *Candida albicans* (n_5) treated with tea tree oil ungerminated cells **(A)** and germinated cells **(B)**



(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).

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