



Original Research Article

Comparative study of the antifungal activity of *Olea europaea* L. against some pathogenic *Candida albicans* isolates in Lebanon

Mohammad Hasan Halawi*, Salwa M. Abdel Rahman and Hoda Yusef

Department of Biological and Environmental Sciences, Faculty of Sciences,
Beirut Arab University, Beirut, Lebanon

*Corresponding author

ABSTRACT

Keywords

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Olive cake,
Olive leaf,
Phenolic content,
Ethanollic extraction,
Aqueous extraction

Olea europaea L. leaves and fruits contain several constituents with considerable pharmacological activities. The present study was designed to evaluate the antifungal activity of Lebanese olive against five strains of *Candida albicans* isolated in a Lebanese hospital. Olive leaves and pomace (cake) samples from three different locations were gathered and extracted differently to obtain three categories of extracts: ethanolic, cold aqueous and hot aqueous. The antifungal activity of these extracts was tested using well-diffusion method with average inhibition zone diameter that reached 27 mm. Two out of the tested extracts; cold aqueous extract of Akkar's cake and ethanolic extracts from Koura's leaves, showed antifungal activity against the growth of all isolates under investigation, with the lowest recorded MIC and MFC of 2.5 and 15 mg/ml, respectively for both extracts. The time kill assay showed that fungal cells died within 6 hours after their treatment with both selected extracts and the decimal reduction time (D-value) determined was 71-72 mins. Total phenolic content was found to be 98.03 GAE mg/g dry matter for ethanolic extracts of leaves from Koura and 91.76 GAE mg/g dry matter for cold aqueous extracts of cake from Akkar, which explains their fungicidal actions and superiority among other tested extracts. The ultrastructure of treated *Candida albicans* with the two selected extracts revealed the presence of deformed cells with disintegrated protoplasm and even ruptured cell wall and cell membrane.

Introduction

Candidemia and invasive candidiasis are a major cause of nosocomial infections, linked to a number of risk factors. The infections with *Candida albicans* in particular, are increasing in frequency. Some species of the genus *Candida* are normally found in the flora of the human skin and mucosa (Eksi, et

al., 2013). They are still the most isolated yeast species regardless of body site and sampling time (Mnichowska-Polanowska, et al., 2013).

Lebanon is famous for its olive production that is considered an important traditional

crop. *Olea europaea* (the olive tree) is a typical Mediterranean species that is well adapted to the climatic conditions in Lebanon and is cultivated in almost all its regions (Abou Zeid, 2007). Olive leaves and fruits contain several constituents with considerable pharmacological activities. The most abundant compound in olive leaf extracts (OLE) is oleuropein, followed by hydroxytyrosol (HT), the flavone-seven-glucosides of luteolin and apigenin; and verbascoside in addition to triterpenes such as oleanolic acid and flavonoids (rutin and diosmin). Extracts of OLE have been found to exhibit antimicrobial effects (Dub & Dugani, 2013). Olive mill wastes cause a major environmental problem in almost all olive oil producing countries (Antoniou, et al., 2002). Thus investigations are necessary to find beneficiary uses of such wastes.

Nystatin and fluconazole are effective agents in the treatment of various fungal infections (Ng & Wasan, 2003). They are the first drugs of choice for the treatment of certain candidiasis (De Aguiar, et al., 2010). Unfortunately, the toxicities caused by polyene antifungals and resistance to fluconazole limit their use (Semis, et al., 2012) and (Robinson, 2014).

The search for novel antimicrobial agents derived from botanical sources never stopped, where plants provide a source of antimicrobial substances needed to manage some of the emerging resistant microbial species (Abreu & McBain, 2012).

Reports on the use of olive extracts against *Candida* spp. in Lebanon are limited. Therefore, the aim of the current study is to achieve a comparative study to determine the anti-candidal effects of various olive tree extracts prepared using different extraction procedures from olive collected from different locations along Lebanon.

Materials and Methods

Sampling

This study was carried out during the period between March 2014 and January 2015. *Candida albicans* were isolated from mouth, vagina, feces, urine and skin of five patients and were coded as S1, S2, S3, S4 and S5, and which were kindly provided by Al Sahel General Hospital, Beirut, Lebanon.

Three districts designated as region 1, 2 and 3 were chosen for olive sampling across Lebanon (Akkar, Koura and Nabatiyeh, respectively). From each area three types of samples were collected, stems, leaves and olive cake.

Olive trees were chosen from a field where they were grown with 20 meters spacing. Samples were mixed from 5 to 100 years old trees, with height varying from 1 to 4 meters, trees where never irrigated or fertilized chemically or organically.

Trees chosen were assessed as disease free and of normal growth, and all trees were of the same local variety of olive trees (baladi). The plant stems were chopped; leaves were separated from branches and stored separately in paper bags, only fully extended green leaves free of pests were chosen. Branches were chosen of 6 to 10 cm long and with soft non woody skin.

Olive cake samples were collected from the same sampling locations and traced to local mills. Samples representing each plant part or cake from each location were mixed and considered as one sample. Leaves, branches and cake were shade-dried at room temperature for 2 weeks and then grounded to a fine powder for ease of extraction of active compounds. The grounded samples were then transported for extraction process at the Laboratory.

Ethanolic extraction

The grounded powder were weighed, soaked in known volumes of 95 % ethanol and allowed to stand for fifteen days with intermittent shaking. Filtration through cotton wool was done to remove coarse particles and finely filtered through filter paper (Whatman® No.1). The filtrate was concentrated by evaporation on a rotary evaporator; concentrated extracts were dissolved in small drops of dimethyl sulfoxide (DMSO) (Cheruiyot & Olila, 2009).

Cold aqueous extraction

The previously prepared grounded powders were soaked in sterile distilled water at room temperature for 15 days, filtered as mentioned previously, then immediately lyophilized and stored at -20 °C. When needed, the extract was redissolved in DMSO and used.

Hot aqueous extraction

The grounded powders were soaked in warm sterile distilled water at 60 °C in a water bath for 45 minutes, allowed to stand for 15 days, filtered as mentioned above, then lyophilized and stored at -20 °C. When needed the extract was redissolved in DMSO and used.

Antifungal activity of olive extracts

The well diffusion assay was used to determine the antifungal effect of various olive extracts against the tested strains of *C. albicans*. A sterile cork borer was used to punch wells of 50 mm diameter on set plates. A 0.5 McFarland standardized fungal suspension was swabbed over the surface of a sabouraud dextrose agar plate (Sia & Yim,

2010). Each well was loaded with 25 µl of one of the prepared extracts and 10 µg of nystatin was used as a positive control. Physiological saline/Dimethyl sulfoxide (DMSO) was used as negative control. The set up was incubated for 24 hours at 37 °C. Twenty four hrs later, the zones of inhibition were measured, and then results were reported in millimeters (Magaldi, et al., 2004). All the tests done for the antifungal evaluation of the prepared extracts were run in triplicates.

Determination of minimal inhibitory concentration of some extracts (MIC)

The MIC of plant extracts that showed antifungal activity in the agar well diffusion assay was evaluated. This test was performed using the broth macro-dilution assay (Eksi, et al., 2013). It was performed using four concentrations of each extract (100, 50, 25, and 12.5 mg/ml), by applying series of dilutions in sabouraud dextrose broth. Then, 100 µl of standardized inoculum (adjusted to 0.5 McFarland) of overnight culture of *Candida albicans* was introduced into every tube, and incubated at 37 °C overnight. The MIC was defined as the lowest concentration of an antimicrobial that inhibited the visible growth of a microorganism after overnight incubation (Andrew, 2001).

Determination of minimal fungicidal concentration of some extracts (MFC)

The minimal fungicidal concentrations (MFCs) of selected extracts were determined by inoculating MIC dilutions onto sabouraud dextrose agar plates and incubated at 37 °C for 24 hrs. After incubation, growth of yeast on solid media indicated that particular concentration of the extract was unable to kill the yeast cells, and so the MFCs were assigned to the lowest

concentration of extracts that resulted in no growth upon sub-culturing(Andrew, 2001).

Determination of the FIC index

The antimicrobial interactive effects of the two most promising extracts (cold aqueous extract of Akkar's cake and ethanolic extracts from Koura's leaves) were assessed using the fractional inhibitory concentration (FIC index). MIC readings were used to quantify the possible interaction between every two antimicrobial extracts(Mun, et al., 2013). FIC indices were calculated with a little modification. When the value of $\Sigma FIC \leq 0.5$, it was noted as synergism and when $\Sigma FIC > 4$ it was noted as the antagonism. A $\Sigma FIC > 0.5$ but ≤ 4 was considered as indifference (Shinde, et al., 2012).

Determination of fungal time kill curve

Inoculated SDB with *C. albicans* isolates and fortified with MFC of extracts prepared from different olive samples were incubated at 37 °C for different time intervals 0, 0.5, 1, 1.5, 2, 4 and 6 hrs(May, et al., 2000). Colony counts were performed by plating 50 μ l aliquots from each culture on SDA plates (dilutions were done when needed) followed by incubation at 37 °C for 24 hrs. CFU on each plate were counted and average CFU/mL was calculated, transformed to \log_{10} and then plotted as \log_{10} CFU/mL vs time (Bubonja-Sonje, et al., 2011). Also, a mixture of the most potent extracts was tested, the time kill curve was traced from the obtained results and compared with the other data acquired from previously tested extracts (Ernst & Rogers, 2005).

Determination of the decimal reduction time

The resistance value (death kinetics) of a *C. albicans* was characterized in terms of decimal reduction time (D-value).

Determination of D-values was by the survivor curves. It was determined from the negative reciprocal of the slopes of the regression lines, using the linear portions of the survivor curves (\log_{10} CFU/mL) versus time of exposure to the extract solution, at constant temperature (Chye & Sim, 2009). The D-value was determined from the inactivation kinetic curve of the most promising extracts given by the equation: $t = D \times (\log N_o - \log N_f) = D \times n$, where D = D-value (min) at specified conditions, N_o = bioburden of the chosen microorganism (*Candida albicans*); N_f = surviving population after an exposure time, t (min), to the selected extract and $n = (\log N_o - \log N_f) = \log_{10}$ reduction of a bioburden(Juneja, et al., 2001).

Transmission electron microscopy

On the basis of MFC values and Time-kill curve data, *Candida albicans* were treated with the most promising antifungal olive extracts for 24 hrs. Cells were fixed using universal electron microscope fixative. Series dehydration steps were followed using ethyl-alcohol and propylene oxide. The samples were then embedded in labeled beam capsules and polymerized. Thin sections of cells were cut using LKB 2209-180 ultra-microtome and stained with a saturated solution of uranyl acetate for half hour and lead acetate for 2 min (McDowell & Trump, 1976). The same procedure was applied to non-treated cells (control). Electron micrographs were taken using a Transmission Electron Microscope (JEM-100 CX Joel), at the Electron Microscope Unit, Faculty of Science, Alexandria University, Egypt.

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS ver.20Chicago, IL, USA).

A significance level of 0.05 was used, below which the results were considered to be statistically significant.

Results and Discussion

Well diffusion assay

Serial dilutions of all stem extracts were tested. Inhibition zones for olive stem extracts were zero against all strains tested regardless of the type of solvent used for extraction, concentration or sample location. Therefore, stem extracts were ignored from any further analysis and were denoted as having no effect against *C. albicans*.

On the other hand, leaf extracts showed more promising antifungal effect. Ethanolic leaf extracts resulted in the largest inhibition zones of all leaf extracts lying between 18 and 27 mm. Hot aqueous leaf extracts, showed a weaker activity than that of ethanolic extracts with inhibition zone diameter lying between 11 and 19 mm. As for cold aqueous leaf extracts, it revealed the weakest activity with inhibition zone diameter of only 7-15 mm.

Among the diameters of inhibition zones related to olive cake; ethanolic extracts proved to be the most potent inhibitor, with inhibition zone diameter between 20-25 mm, cold aqueous extracts showed an inhibition zone diameter between 7-16 mm and hot aqueous extracts had inhibition zones of 7-14 mm in diameter.

Overall, ethanolic extracts of both leaves and cake showed the highest activity and were followed by hot aqueous leaf extracts (Table 1). All strains tested showed comparable susceptibility. Figure 1 shows that when the overall activity of extracts of a certain region were to be compared, differences were insignificant.

MIC and MFC values

The minimum inhibitory concentration and minimum fungicidal concentration were studied and listed in Figure 2 and it was found that: Cold aqueous cake extracts were found to have the most significant MIC values against *C. albicans* (2.5 – 30 mg/ml). Extracts from Akkar (region 3) being the most significant of all with MIC of 2.5 mg/ml (against some strains). Hot aqueous cake extracts of Koura (region 2) had intermediate MIC of 20 mg/ml.

Considering leaf extracts, ethanolic extracts of region 2 (Koura) had the most significant MICs of 15 mg/ml. Concentrations used failed to give results within range tested for other extracts (hot aqueous leaf and ethanolic cake). Cold aqueous cake extracts were found to have the most significant MFC values against *C. albicans* (2.5- 30 mg/ml). Extracts from Akkar (region 3) being the most significant of all with MFC of 2.5 mg/ml (against some strains). Hot aqueous extracts of cake from Koura (region 2) had an MFC of 20 mg/ml.

Considering ethanolic extracts of leaves from region 2 (Koura) had the most significant MFC of 15 mg/ml. Concentrations used failed to give results within range tested for other extracts (hot aqueous leaf and ethanolic cake).

The time kill curves revealed that the association of cold aqueous extracts of cake from Akkar with fluconazole induced a slight reduction in viable count compared to fluconazole alone; however its association with nystatin produced a significant reduction in killing time, which was shorter than that recorded for the extract alone or nystatin alone. This synergic effect produced a 99.9% kill of all viable cells in 4 hours (Figure 3).

Ethanollic extracts of leaves from Koura combined with fluconazole produced a single \log_{10} reduction in the sixth hour if compared to fluconazole alone. However, its association with nystatin produced a synergic effect, with a 99, 9% kills of all viable cells in 3 hours (Figure 4).

It was revealed that cold aqueous extracts of cake from region 3 together with ethanollic extract of leaves from region 2 expressed an antagonistic effect, cells of *C. albicans* were able to divide in a manner similar to growth controls (figure 5), this was assessed when FICI was calculated, where a concentration of 100 mg/ml failed to produce growth inhibition and FICI value was found to be greatly higher than 4.

From the prior findings of time kill assay the decimal reduction time for both selected extracts was calculated using survival curves, where the D-value for ethanollic extract of leaves from region 2 was found to be 72 mins and that of cold aqueous extract of cake from region 3 was found to be 71 mins with a similar potency of both extracts to reduce viable cells count of *C. albicans* viable cell in a close time interval.

For a better understanding and interpretation of the results, total phenolic content was calculated based on a standard gallic acid calibration curve. The two selected extracts' total phenolic content exceeded those of the other extracts. Ethanollic extracts of leaves from Koura had a total of 98.03 GAE mg/g and aqueous extarcts of cake from Akkar had 91.76. All the other extracts had values ranging between 22.3 and 82.3 GAE mg/g (Figure 6).

The ultrastructures of treated *C. albicans* displayed noticeable morphological changes. Ethanollic extarcts of olive leaves from Koura caused a deformation in cell shape and

protoplasm disintegration (Figure 7). Cold aqueous extracts of olive cake from Akkar caused a disruption in the cell wall and cytoplasmic membrane of the cells which led to protoplasm leakage along with vacuolation (Figure 8).

Many natural agents like plant extracts are traditionally used against various ailments. Some of the plant products were found effective against *Candida* and other infection causing fungi (Devkatte, et al., 2005). The use of plant derived products as diseases control agents have been studied, since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Rana, et al., 2011).

Some researchers reported that most phenolic derivatives of olive have a fairly broad spectrum of action which is fungistatic (Al-Mughrabi, et al., 2001). Olive cake has been found to possess antimicrobial characteristics (Winkelhausen, et al., 2005) and olive leaf extract has been demonstrated to be effective in treating fungal outgrowths (Bruno, 2004). The results of (Seshathri, 2012) revealed that *Olea europaea* extracts showed maximum inhibition of oral infection caused by *C. albicans* when compared to other plant extracts.

In the present investigation, the most active olive extracts against *C. albicans* were ethanollic leaf and cake extracts, which both exhibited the strongest inhibitory effect against the fungal strains under test with an average inhibition zone diameter of 22 mm for both extracts. Every extract type and each plant sample collected from a certain region produced different inhibition values. These are not surprising, considering that different extractive methods were applied, involving higher temperature; and that aqueous extracts are more polar than the ethanollic ones. On the other hand, the

influence of the sample origin, in terms of both geographic provenance and other factors, cannot be excluded (Pereira, et al., 2007). A study performed by (Kännaste, et al., 2013) demonstrated a huge variability in chemical contents and signatures in plants across and within geographical sites, which was associated with differences in nutrients, water availabilities, and chemotypic differences.

The minor differences in strain sensitivity are normal to be observed, as *Candida* strains are known to respond differently to the same antifungal treatment (Ota, et al., 2001).

While ethanolic leaf extracts had the lowest MIC out of all leaf extracts (15 mg/ml recorded for region 2), ethanolic cake extracts did not exhibit the same effect. Cold aqueous cake extracts had much lower average MIC (2.5 mg/ml recorded for region 3) than that of ethanolic extracts (20 mg/ml). Studies suggested that a single susceptibility test such as a MIC or the measurement of inhibition zones, does not provide a comprehensive profile of the antimicrobial activity of an antimicrobial agent. A combination of tests is desirable in a sense that MICs and zones of inhibition do not always correlate with each other (Yin, et al., 1999).

MIC and MFC values for selected extracts against tested *C. albicans* isolates showed a good correlation of results. There was a significant change in MFC values between extracts based on the extraction solvent, where hot aqueous cake extracts and cold aqueous leaf extracts showed MIC and MFC values which were out of the range of test (≤ 35 mg/ml). Some ethanolic leaf extracts, cake extracts and hot aqueous leaf extracts failed to give MIC and MFC values recognizable by test parameters. Results

agreed with those conducted priorly by (Sudjana, et al., 2009) that found *Candida* very poorly susceptible to high concentrations of some olive leaf extracts (50% v/v), and who failed to record an MFC.

Obied et al. (2007) observed no antimicrobial activity of olive mill wastes against *C. albicans* which corroborates to this study findings. However, Obied et al. (2007) tested the methanolic extracts of olive that acted similarly and matched the ethanolic extracts prepared in the current study, which could explain the anti-candidal activity resulting from cold aqueous cake extracts found in current study.

The time kill curve study showed that both selected extracts of ethanolic leaves and cold aqueous cake showed excellent and rapid fungicidal activity within 6 hrs. Erturk et al. (2006) reported that the ethanolic extracts of some plants leaves may show higher inhibitory activity against *C. albicans* than the standard antifungal nystatin. Time kill curves analysis also revealed a synergic mode action between extracts and selected antifungal drugs; while the synergic effect was weak when each of the extracts were associated with fluconazole, it had significant inhibitory effect when associated with nystatin.

When both extracts were tested alongside, an antagonistic mode of action was revealed where *C. albicans* cells continued to grow in a manner comparable to control, knowing that these extracts were just crude extracts of uncertain composition, its components can have synergistic or antagonistic effects (Al-Hussaini & Mahasneh, 2009). The antagonistic and synergic effects were also calculated related to MIC values, and results agreed those concluded from the time kill curve.

Table.1 Mean inhibition zone diameter (mm) as affected by regional variations among olive extracts tested against *Candida albicans* strains

Region Extract Type	Mean of inhibition zone diameter (mm)			
	1	2	3	Mean of means
Ethanollic leaf	22 _a	22 _a	21 _a	22 _a
Ethanollic cake	21 _a	22 _a	22 _a	22 _a
Hot aqueous leaf	13 _a	18 _b	16 _c	16 _b
Hot aqueous cake	12 _a	10 _b	12 _a	11 _c
Cold aqueous leaf	10 _a	11 _a	10 _a	10 _c
Cold aqueous cake	11 _a	9 _b	13 _a	11 _c
Mean of Means	15 _a	15 _a	16 _a	-
F	0.621			

Means in the same row and sub-table not sharing the same subscript are significantly different at $p \leq 0.05$

Means of Means in the same column or row not sharing the same subscript are significantly different at $p \leq 0.05$

Region 1: Rchaf, Region 2: Koura, Region 3: Akkar.

Figure.1 Mean inhibition zone diameter recorded for tested extracts of leaves and cake (collected from different regions) against *Candida albicans*

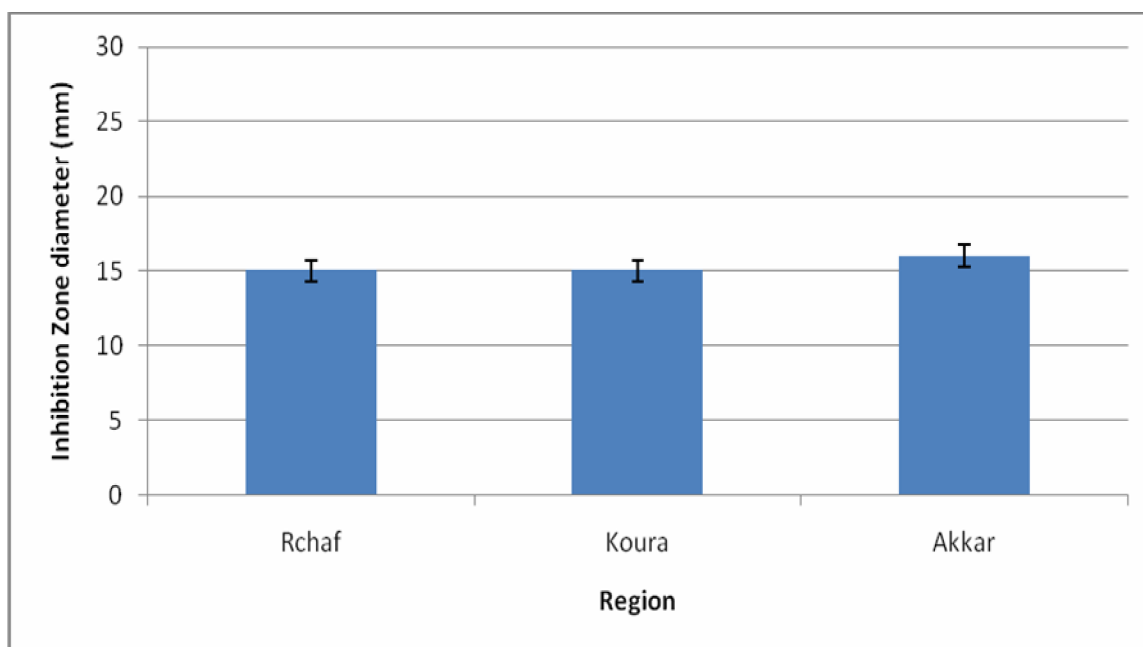


Figure.2 Minimum inhibitory concentration and minimum fungicidal concentration of tested olive extracts against strains of *Candida albicans*

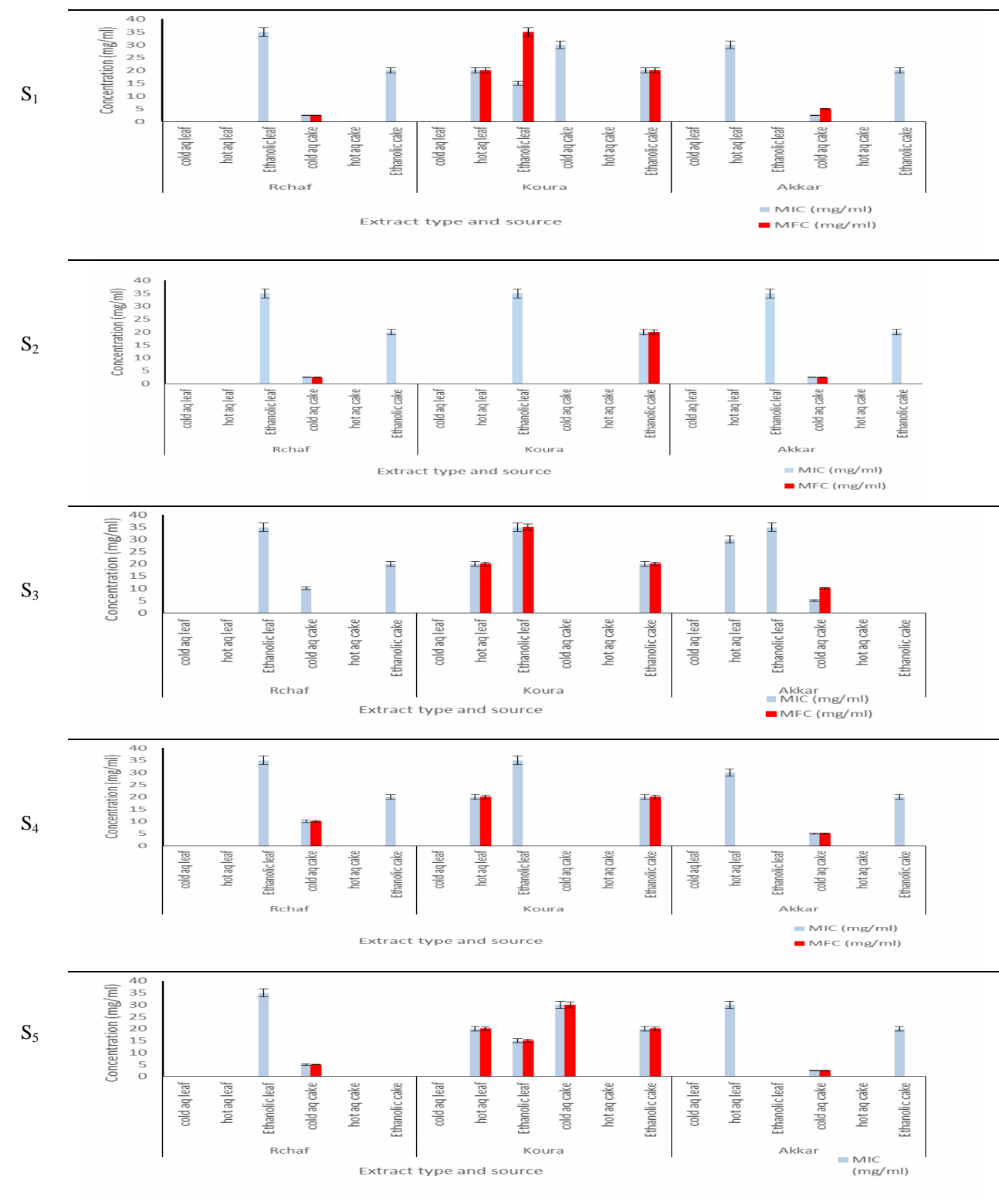


Figure.3 Time kill curve of *Candida albicans* S1 treated with EI2

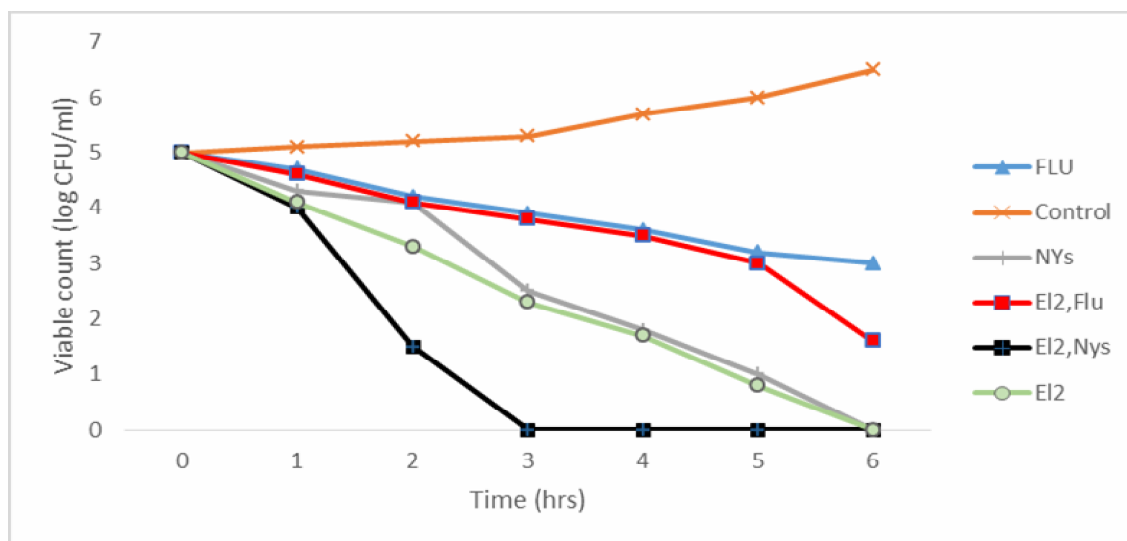
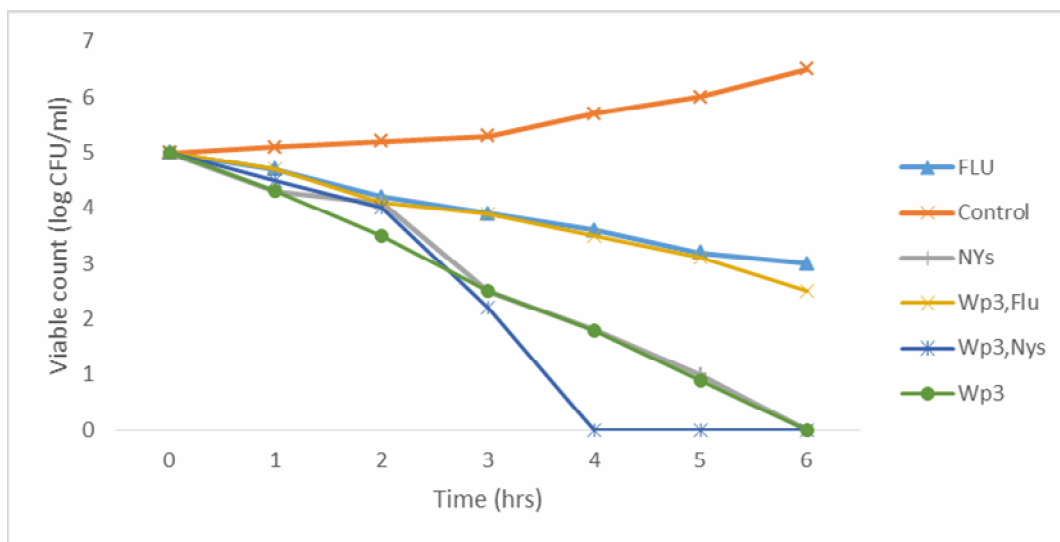
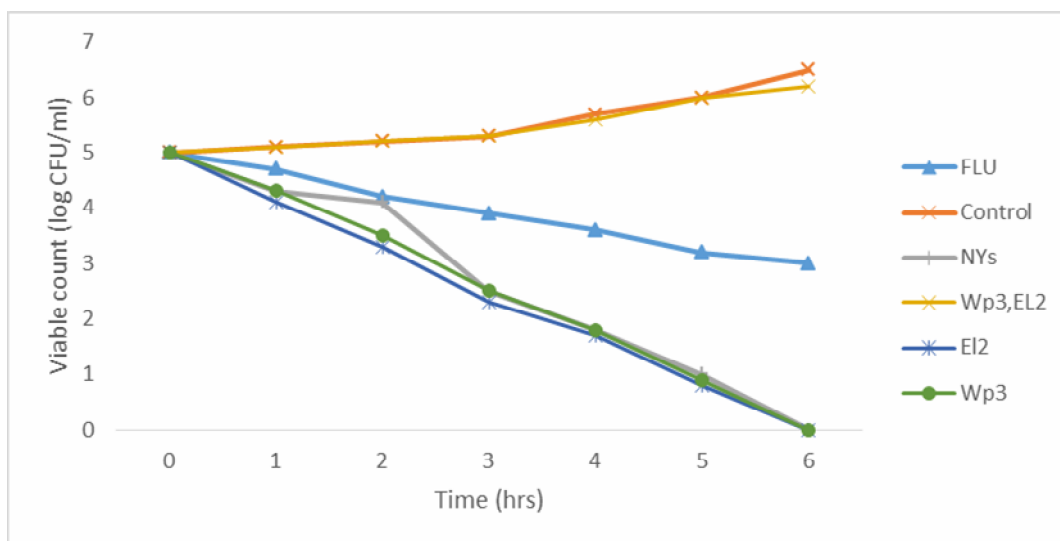


Figure.4 Time kill curve of *Candida albicans* S₁ treated with Wp3



FLU: Fluconazole, Control: Growth of untreated cells, NYS: Nystatin, Wp3/Flu: Cold aqueous extracts of pomace from region 3 and fluconazole, Wp3/Nys: Cold aqueous extracts of pomace from region 3 and nystatin, Wp3: Cold aqueous extracts of pomace from region 3.

Figure.5 Time kill curve of *Candida albicans* S₁ treated with EL2 and Wp3



FLU: Fluconazole, Control: Growth of untreated cells, NYS: Nystatin, Wp3/EL2: Cold aqueous extracts of pomace from region 3 and ethanolic extracts of leaves from region 2, EL2: Ethanolic extracts of leaves from region 2, Wp3: Cold aqueous extracts of pomace from region 3.

Figure.6 Total phenol content of olive extracts as gallic acid equivalent mg/g of dry matter

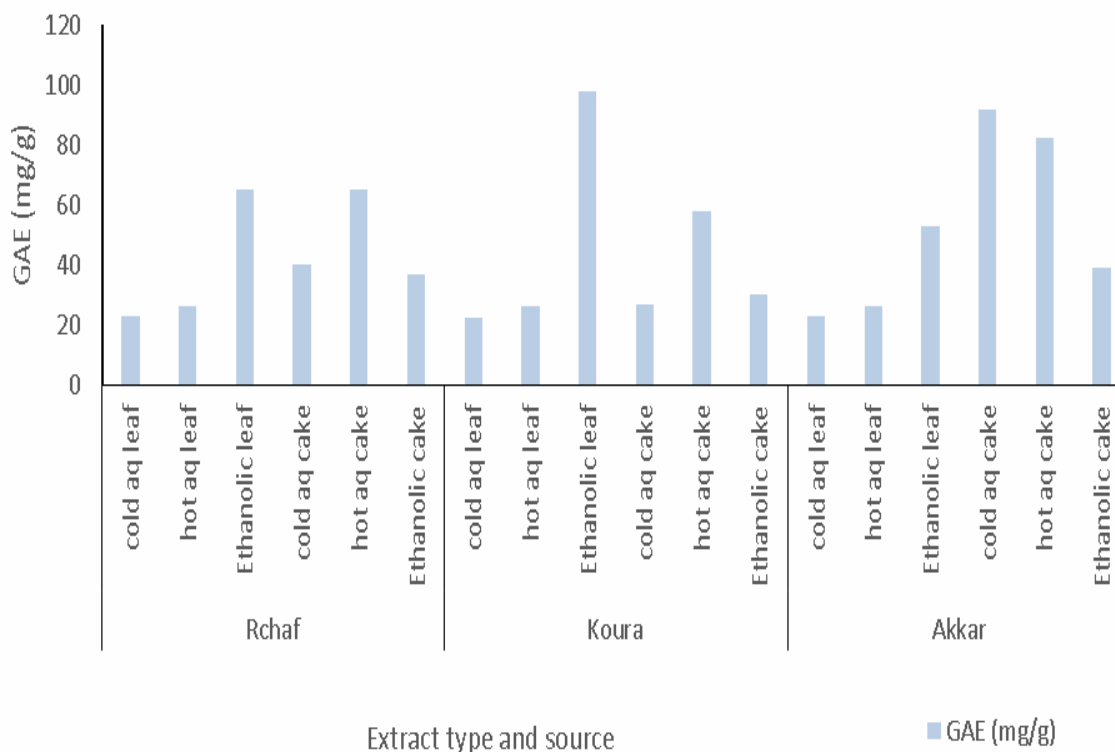
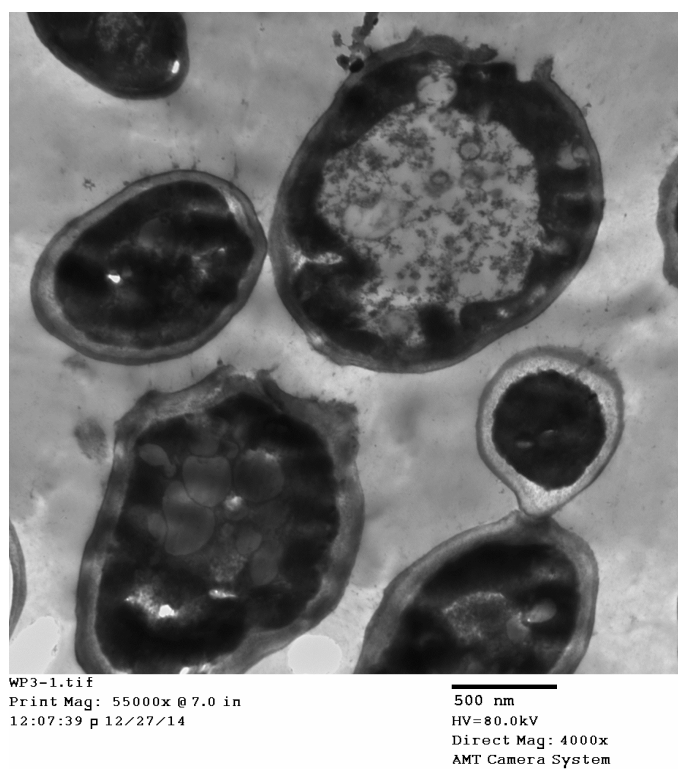


Figure.7 Transmission electron micrographs of *Candida albicans* S₁ treated with ethanolic extract of leaves from Koura



Figure.8 Transmission electron microscopy of *Candida* sp. treated with cold aqueous cake



D-values of both most active extracts were 72 and 71 mins. It was normal to have DRT as high as 17 hrs given that it is well known that a lower MIC, MBC or MFC (in case of fungi) does not necessarily demonstrate a lower DRT value (Chye & Sim, 2009)

It is well stated that antimicrobial and antioxidant activities of plants were correlated with total phenolic components of plant extract (Katalinic, et al., 2013)(Sengul, et al., 2011)(Da Silva, et al., 2006).

The current investigation led to the conclusion that *Olea europaea* leaves and cake extracts are potential sources of novel and natural agents that could be used in the treatment or prevention of important fungal infections caused by *C. albicans*. Regional differences did not prove to be of high significance. Olive stem extracts did not show any significant antifungal activity. Further chemical and biochemical analysis should be done to isolate the active ingredients behind the antifungal activity found in order to concentrate them and produce them in a proper pharmaceutical form.

The possible future uses of olive extracts may hold various advantages. It may offer an environmental solution to the problem of olive solid wastes produced from milling in the form of olive cake. The antifungal properties of leaf and cake extracts can have broad uses, from plant-derived fungicides to antifungal drugs for treatment of candidiasis in humans or even as a prophylactic supplement in daily diet.

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