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# Evaluation of the Inhibitory Effect of Marine Macroalgal *Ulva lactuca* extracts on Oral *Candida* spp. Biofilms Formation

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**ABSTRACT**

The increasing clinical and microbiological resistance of *Candida* species towards several commonly prescribed antifungal agents has stimulated the search for new active antifungal compounds from natural resources. The most important virulence factors of pathogenic *Candida* species are biofilm formation and hyphal development, making treatment very challenging due to the spread of resistant *Candida* isolates. This study was designed to explore the potential of different types of *Ulva lactuca* extracts on the growth of *Candida* spp., and evaluate the morphological alterations and time-kill as well. Methanol was the best solvent for extracting bioactive components from the examined algae *Ulva lactuca* (Chlorophyta) exhibiting the highest antifungal effect, followed by ethanol and chloroform. The minimum inhibition concentration (MIC) of *U. lactuca* methanol extract (ULME) was 62.5mg/ ml, and the minimum fungicidal concentration (MFC) was 125mg/ ml against all the tested oral *Candida* spp. Different concentrations of ULME (1xMIC, 2xMIC and 4xMIC) influenced the micromorphology of the tested oral *Candida* spp. and had an impact on biofilm formation. In the time-kill curves, ULME showed a concentration-dependent fungicidal effect. The cytotoxicity results showed that ULME has no significant toxic effects against normal human lung fibroblast cells (MRC-5 cell line). Moreover, the major phytochemical compounds were fatty acids, identified and quantified by using Gas Chromatography-Mass Spectrometry (GC/MS). In conclusion, ULME may be an alternative to conventional antifungal drugs for the treatment of oral candidiasis.

## INTRODUCTION

Fungal infections are the fourth leading cause of hematogenous infections. The most common involved fungi are *Candida* species (commensal microorganisms) that are known for their potential to infect humans superficially and systemically **(Tsai *et al.*, 2014)**. The emergence of non-*albicans Candida* spp. produces significant infections that are difficult to treat the human populations all over the world. The impaired competitive of the host microbiota is a prerequisite for candidiasis, and this facilitates *Candida* growth **(Cavalheiro & Teixeira, 2018)**.

One distinct feature of *Candida* species pathogenicity is their ability to form biofilms, which may help fungi retain their pathogenic role by evading host immune mechanisms and resisting antifungal treatment due to the presence of the matrix, which restricts drug penetration by forming a diffusion barrier **(Kojic & Darouiche, 2004**; **Nett *et al.*, 2011; Cavalheiro & Teixeira, 2018)** and withstanding competitive pressure from other organisms. Biofilm-forming fungal infections are very difficult to treat; hey often involve increased drug-resistance phenotypes **(Pierce *et al.*, 2013)**. The available, synthetic antifungal medicines are very toxic to host tissues, resulting in severe effects. One of the hurdles to the development of new antifungal agents that are active against biofilms is the scarcity of tests for either the characterization or identification of new compounds with activity towards biofilms **(Oshiro *et al.*, 2019)**. Unfortunately, the treatment options are highly limited, as there are few chemical classes represented by existing antifungal drugs **(Cowen *et al.*, 2015)**. The rising prevalence of drug-resistant pathogens as well as the toxicity of conventional antifungal drugs have focused the emphasis on the antimicrobial activity of natural materials **(Paramythiotou *et al.*, 2014)**.

Marine organisms produce a wide range of pharmacologically active compounds, including anticancer, antibacterial, antifungal, antiviral, anti-inflammatory among others; they are potential sources of new therapeutic agents. In a competitive and hostile environment, they survive and live within complex communities and in close interaction with others, producing complex secondary metabolites in response to ecological pressures such as competition for space, predation and tide variations. Some of these compounds are antimicrobials, which prevent or limit the development and growth of other competitive microorganisms **(Pérez *et al.*, 2016)**. Marine natural products are currently recognized as the most important source of bioactive substances and drug leads **(Wali *et al.*, 2019)**. These natural drugs are less expensive and less harmful to host tissues **(Rajeshkumar & Sundararaman, 2011)**. The antifungal activity of seaweeds extracts and isolated compounds has not been substantially examined since the greater emphasis has been directed to pathogenic bacteria, which is so far more explored **(Mayer *et al.*, 2013)**.

*Ulva lactuca*, often known as sea lettuce, has long been used as a meal and a traditional medicine to cure helminthic infections, fever, urinary disorders and dropsy. The antimicrobial action of *U. lactuca* is caused by the acrylic acid typically found in the algae **(Shokripour *et al.*, 2020)**. **Caf *et al*. (2015)** reported that the green algae are rich in palmitic acid (C16:0). In addition, *Ulva* contains phenolic, chlorophyllic and carotenoids, serving as active free radical scavengers **(Cesário *et al.*, 2018)**. The macroalgae *Ulva lactuca* has been used as a natural source extract with high bioactivity (**Pappou *et al.*, 2022)**. However, *Ulva Lactuca* L. seaweed extract can be used as an antifungal agent in the preparation of eco-friendly disinfectants **(Krishnamoorthi & SivaKumar 2019)**.

The aim of the present investigation was to evaluate the inhibitory effect of the most potent *U. lactuca* solvent extract on *Candida* spp. biofilm and evaluate the morphological alterations and time-kill as well.

## MATERIALS AND METHODS

### Marine algal sampling

Samples of *Ulva* were collected from Ras El-Adabiya located on the western shore of Suez Bay (Gulf of Suez) at latitude and longitude 29.681737, 32.508970, respectively, during summer 2014 from the intertidal zone of the Gulf of Suez (Fig. 1a, b).


**Fig. 1. (a)** Google earth image showing the location of the collection site: Ras El-adabiya on the western shore of Suez Bay according to its topography at lat., long. (29.681737, 32.508970), respectively. **(b)** Google earth plane satellite image showing the collection site: Ras El-adabiya at lat., long. (29.681737, 32.508970), respectively.

To eliminate foreign particles, sand particles and epiphytes, the samples were thoroughly rinsed with seawater. Afterwards, the alga species was identified according to **Joska, (1992)**. Samples were then placed in an icebox and immediately brought to the laboratory and washed thoroughly with distilled water to remove the salts on the surface of the samples. They were placed over blotting paper. The air-dried samples were placed in a 50°C oven, pulverized in the grinder, then sieved through a screen with mesh size of 0.5 mm. The powdered substance was stored in airtight plastic bottles at room temperature for further studies **(Barot *et al.*, 2016)**. All samples collected were dried and transformed into a powder form.

**Algal extracts**

Ten grams of powdered algal sample was extracted by methanol, ethanol, chloroform according to **Cho *et al.* (2007)**; aqueous extracts were prepared according to **Jiménez *et al.* (2011)**. The resulting extracts were concentrated to dryness under reduced pressure at 40– 45°C with a rotary evaporator and stored at 4°C in air-tighted glass bottle for further assay. All solvents used were of analytical reagent grade, and they were obtained from Sigma Chemical Co. (USA).

**Quantitative determination of some phytochemicals**

Quantitative determination was carried out at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt. The following parameters were evaluated as follows: total protein by micro-Kjeldahl's **(James, 1995)**; total carbohydrates by phenol-sulfuric acid method **(Dubois *et al.*, 1956)**; total lipids using phosphovanillin method **(Barnes & Blackstock, 1973)**, and phenols contents were determined according to **Singleton *et al.* (1999)**.

**Fungal strains and culture conditions**

Four *Candida* spp. used in the current study were isolated from the oral cavity of diabetic patients and identified using Integral Yeast System Plus as previously published in the study of **Moustafa *et al.* (2015)**. The *Candida* strains were individually cultured on Sabouraud Dextrose (SD) broth medium (Difco) at 37°C for 48h before inoculation for assay. To prepare the inoculums, 24h old cultures of each *Candida* spp. was inoculated into 5ml SD broth medium, and turbidity of suspension was adjusted to obtain $O.D_{(600nm)}$ ~0.5, which is equivalent to $10^8$ CFU/ml and spectrophotometric readings using spectronic 21 spectrophotometer (Bauch and Lomb, New York, USA) **(Souza *et al.*, 2007)**.

**Antifungal assay**

The antifungal activity of algal extract was determined using the agar diffusion method **(Suay *et al.*, 2000)**. Using a sterile cotton swab, *Candida* spp. inoculum were uniformly spread on SD agar plates and allowed to dry at room temperature, wells were formed on the surface of agar medium with 6mm cork borer. Each well in the plate was filled with 50µl of the tested algal crude extracts. The plates were incubated at $37^oC$ for 24- 48h. After incubation, the plates were examined for zone of inhibition, and the diameters of the zones were measured in millimeters. All assays were performed independently in triplicates, and the mean result were calculated; in addition, fluconazole (100mg $ml^{-1}$) and DMSO were employed as positive and negative controls, respectively.

**Minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC)**

The minimum inhibitory concentration (MIC) was applied on all crude extracts to demonstrate their efficacy against all tested *Candida* species by the agar well diffusion method. It was determined using the two-fold serial dilution method **(Baharuddin *et al.*, 2015)**. Crude extracts that had an effect against *Candida* spp. were serially diluted using SD broth medium as a diluent to yield final crude extract concentrations ranging from 250 mg/ml to 0.06 mg/ml. The tubes were inoculated with *Candida* suspension (20µ/ml broth) homogenized and incubated at 37$^{o}$C for 48h. MIC value was calculated as the lowest concentration of extract that inhibited the growth of the tested *Candida* spp. **(Patra *et al.*, 2009)**. Sabouraud Dextrose broth medium was used for the positive and negative controls. Following an overnight incubation for the MIC determination, 50µl of each tube which indicates no growth for all respective *Candida* sp. were subcultured onto SD agar plates. The plates were incubated at 37°C for 24 to 48h until visible growth was observed. The MFC value was determined as the concentration with no growth or fewer than 3 colonies were obtained to a killing activity of roughly 99:99.5 **(CLSI, 2002 – M38)**.

**Determination of the percentage inhibition of diameter growth (PIDG)**

Different concentrations of the most potent algal crude extract exhibiting an inhibition ability against all tested *Candida* spp. were prepared, then compared with commercial mouth rinse of 0.1% CHX as a positive control **(Himratul-Aznita *et al.*, 2011)**. PIDG values were evaluated upon applying the following equation:

$$\text{PIDG (\%)} = \frac{\text{Diameter of sample} - \text{Diameter of control}}{\text{Diameter of control}} \times 100$$

**Antibiofilm assay**

The strains were characterized for biofilm formation using crystal violet protocol, with slight modifications made by **Shin *et al.* (2002)**. A set of polystyrene tubes containing 9ml SD broth medium, and 1ml of MIC of the most potent crude extract that exhibit its ability against all the tested *Candida* spp. were placed to each tube. A 100µl of each fresh *Candida* species culture grown for 24h at 37°C was separately added, after which samples were incubated for 48 and 72h at 37°C, respectively. *Candida* biofilms were examined by a scanning electron microscopy (Model: JSM-5500 LV; JEOL Ltd Japan), using high vacuum mode at the laboratory center, Faculty of Science, Ain Shams University, Cairo, Egypt.

**Micromorphology of *Candida* spp. using different MIC doses of algal extract**

Microculture on glass slide in Petri dish (moist chamber) was used to assess the action of the most potent algal crude extract on the micromorphology of the tested *Candida* species

**(Alves *et al*., 2013)**, with some modifications. Melted cornmeal agar-Tween 80 culture medium (CTA) (HiMedia Lab., India) was added to sterile tubes without extract and other containing most potent extract at concentrations corresponding to the MIC, 2xMIC, and 4xMIC. After mixing, culture medium was poured. After solidification, a deep cut (horizontal furrow) was made in CTA. Then, a flamed sterile cover slip was placed over the line of the inoculum and incubated at 35°C for 48 hours. The plates were incubated at 35ºC for 24- 48h. The slides were examined with a light microscope at 400x magnification **(Gatica *et al*., 2002)** to determine whether some *Candida* species characteristic structures, such as blastoconidia, pseudohyphae and chlamydoconidia were present. Finally, the observation was interpreted according to **Koehler *et al*. (1999)**.

**Time- kill assay**

The kinetic assays of microbial killing of tested oral *Candida* species in the presence of the most potent algal crude extract were carried out according to **Klepser *et al*. (1998)**. One ml of *Candida* suspension (1-5 x $10^6$ CFU/ ml) was added to 9ml SD broth **(Oxoid)** with or without the algal crude extract at various appropriate concentrations (1x, 2- and 4-times MIC). Cultures were incubated at 37°C for various time periods (0, 2, 4, 8, 12 and 24h). Then, 100µl was removed from each solution and serially diluted in sterile distilled water. An aliquot of 100µl of each dilution was plated on SD agar. Finally, the plates were incubated at 37°C for 24- 48h, and CFU/ml were counted. When fewer than 1000 CFU/ml was expected, 10µl samples were directly plated on SD agar without dilution. The experiment was carried out in duplicate, and the method's minimum detection limit is 100CFU/ ml. $Log_{10}$ CFU/ml versus time were plotted to compare the rate and the extent of antifungal activity at various concentration of the most potent algal crude extract. Fungicidal activity was defined as $\geq 3$ $log_{10}$ (99.9%) decrease in CFU/ml from the initial inoculum. A lower activity was considered as fungistatic **(Ernst *et al*., 2002)**.

**Cytotoxicity assay**

*In vitro* cytotoxicity was conducted and assessed for the most potent *U. lactuca* crude extract that exhibit its ability against all the tested *Candida* spp. at Bioassay - Cell culture, Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar university, Cairo, Egypt. Cytotoxic assay against normal human lung fibroblast MRC-5 cell line using MTT assay **(Mosmann, 1983**; **Gomha *et al.*, 2015)**.

**GC-MS analysis**

The GC-MS analysis was performed at Central Lab., Faculty of Science, Ain Shams University, Cairo, Egypt. The gas chromatography using Gas Hewlett Packard HP-5890 series II equipped with split/ split less injector and a capillary column (30m, 0.25mm, 0.25µm film thickness) fused with phenyl polysilphenylenesiloxane. The column temperature was programmed to rise from 70 to 280°C over a period of 20min at a rate of

20°C/ min. The injector temperature was set to 280°C, and injection volume was set at 5µL. Helium (1 mL/min) was used as the carrier gas. The phytochemical components were identified by diluting the most powerful crude extract with DMSO. Total running time was 48min. The compounds were tentatively identified based on a comparison of their respective retention time and mass spectra with those of the NIST, Willy library data of the GC/MS system **(Pandey *et al.*, 2010)**.

**Statistical analysis**

SPSS software **(SPSS, 2012)** was used for the statistical analyses. Acquired data were statistically analyzed to evaluate the level of significance using one-way analysis of variance (ANOVA) at the probability level of $P \leq 0.05$. A post-hoc test was applied according to Duncan's test when differences are significant.

## RESULTS

The collected samples were morphologically identified as *U. lactuca* according to **Abbott and Hollenberg (1976)**, **Aleem (1978)** and **Joska (1992)**. The effect of its extracts was evaluated on four strains of *Candida* spp. including *C. albicans, C. tropicalis, C. krusei* and *C. glabrata*. Marine products hold great promise as a source of active compounds that can be used to treat a variety of diseases. The total lipids, carbohydrates and proteins that was recorded for *Ulva lactuca* recorded the values of 41.1%, 24.9% and 14.4%, respectively (Fig. 2). The effect of different solvents extracts of *U. lactuca* against *Candida* spp. showed that methanol extract had significant antifungal effect than other solvent extracts against all *Candida* spp. tested (*C. albicans, C. tropicalis, C. krusei* and *C. glabrata*), with inhibition zones of 45.00±2.4, 35.00±0, 32.00±2.1 and 30.00±2.5mm, respectively, while ethanol extract had equal effect on all *Candida* spp., except for *C. tropicalis* that had no effect. Whereas, chloroform extract equally affected *C. albicans* and *C. glabrata* growth and had no effect on *C. krusei*, with significant differences at $P < 0.05$. Moreover, hot and cold water extracts had no effect as shown in Fig. (3).

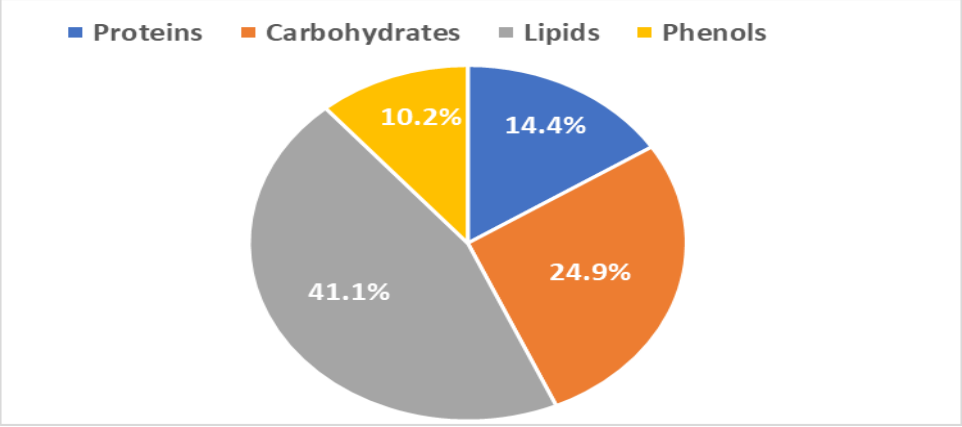



**Fig. 2.** Quantitative determination of total proteins, carbohydrates, lipids and phenols

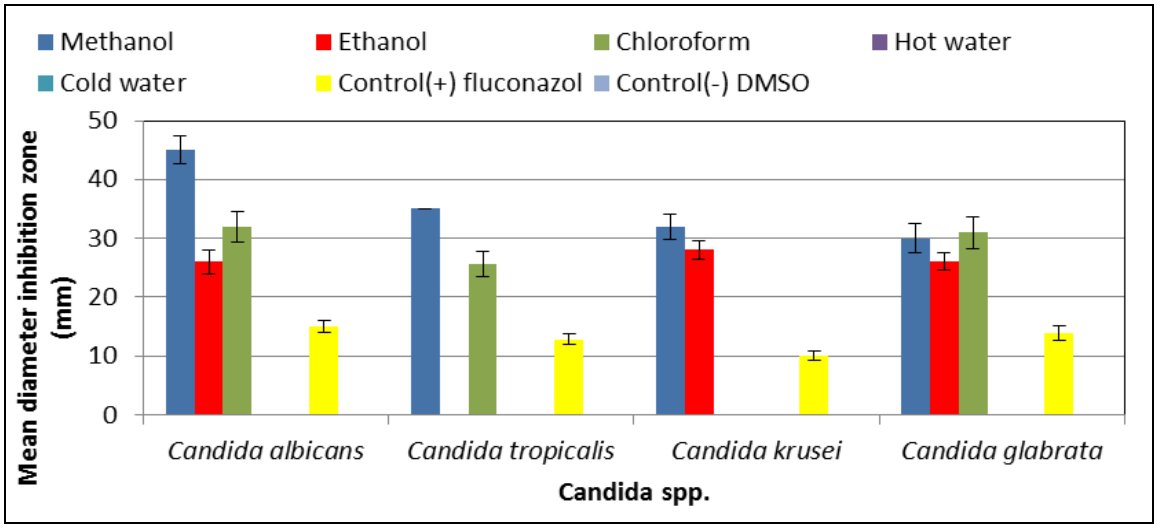



**Fig. 3.** Antifungal activity of *U. lactuca* crude extract with different solvents against the tested *Candida* spp.

Most of *U. lactuca* extracts inhibited the growth of *Candida* spp. The MIC & MFC were not detected in the cases of ethanol with *C. tropicalis* and chloroform with *C. krusei*. MIC values in all others were detected with 62.5mg/ ml, and in the case of MFC values between 125 & 250 mg/ml compared with positive control 125/250 with all *Candida* spp. (Table 1). In general, The MIC/MFC ratio was 62.5/125 in all treatments, except for ethanol with *C. krusei* and *C. glabrata* whose ratio was 62.5/250.

**Table 1.** Values of MIC and MFC (mg/ml) for *U. lactuca* crude extract with different solvents against the tested *Candida* spp.

| Ulva extract | *C. albicans* | | | *C. tropicalis* | | | *C. krusei* | | | *C. glabrata* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MIC | MFC | MFC/MIC | MIC | MFC | MFC/MIC | MIC | MFC | MFC/MIC | MIC | MFC | MFC/MIC |
| **Methanol** | 62.5 | 125 | 2 | 62.5 | 125 | 2 | 62.5 | 125 | 2 | 62.5 | 125 | 2 |
| **Ethanol** | 62.5 | 125 | 2 | NA | NA | -- | 62.5 | 250 | 4 | 62.5 | 250 | 4 |
| **Chloroform** | 62.5 | 125 | 2 | 62.5 | 125 | 2 | NA | NA | -- | 62.5 | 125 | 2 |
| **Fluconazole** | 125 | 250 | 2 | 125 | 250 | 2 | 125 | 250 | 2 | 125 | 250 | 2 |

The determination of the PIDG for all of the tested oral *Candida* spp. revealed that ULME crude extract inhibited more *Candida* spp. (Tables 2, 3) than CHX (a positive control used in this study), which is a common antimicrobial agent in a commercialized oral rinse. The ULME with PIDG values of more than 50% at 25mg/ ml for *C. albicans* and *C. krusei* outstrips the used positive control (chlorhexidine 0.1%). The PIDG values were more than 50% for *C. tropicalis*, *C. krusei* and *C. glabrata* while recording more than 100% for *C. albicans* at 50mg/ ml. However, the PIDG values were more than 150% at 100mg/ ml for all the tested oral *Candida* spp., except *C. glabrata* (114%). The percentage of inhibition of various concentrations of ULME towards the tested oral *Candida* spp. is illustrated in Fig. (4).

**Table 2.** Effect of different *Ulva lactuca* methanol crude extract concentrations against the tested *Candida* spp.

| ***Candida* species** | **Diameter of inhibition zones (mm)** | | | | |
|---|---|---|---|---|---|
| | ***Ulva lactuca* methanol extract (mg/ml)** | | | | **CHX (%)** |
| | **25** | **50** | **75** | **100** | **0.1** |
| ***Candida albicans*** | 20.67 | 27.67 | 32 | 45 | 11.33 |
| ***Candida tropicalis*** | 19.67 | 25 | 29.67 | 35 | 13.33 |
| ***Candida krusei*** | 18.67 | 22.67 | 28.67 | 32 | 12.33 |
| ***Candida glabrata*** | 17 | 22 | 26.67 | 30 | 14 |

**Table 3.** PIDG of oral *Candida* spp. towards different methanol extract concentrations of *Ulva lactuca*

| ***Candida* species** | ***Ulva lactuca* methanol extract** | | | |
|---|---|---|---|---|
| | **25(%)** | **50(%)** | **75(%)** | **100(%)** |
| ***Candida albicans*** | 82.43 | 144.21 | 182.43 | 297.17 |
| ***Candida tropicalis*** | 47.56 | 87.54 | 122.58 | 162.56 |
| ***Candida krusei*** | 51.41 | 83.86 | 132.52 | 159.52 |
| ***Candida glabrata*** | 21.42 | 57.14 | 90.5 | 114.28 |

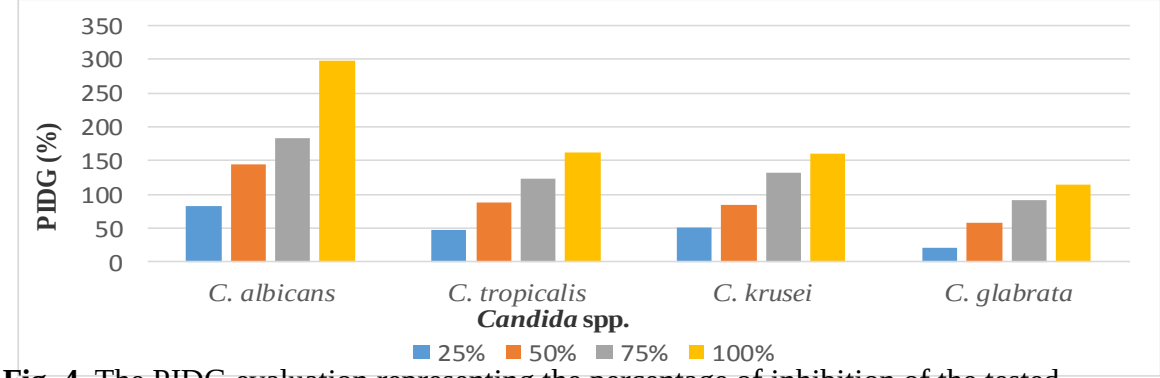



**Fig. 4.** The PIDG evaluation representing the percentage of inhibition of the tested *Candida* spp. upon exposure to ULME

The antibiofilm activity of ULME was determined against *Candida* strains and observed by SEM, as shown in Photo (1A) showing the biofilm of *C. albicans* in the absence of ULME (control) after 48h; *C. albicans* biofilm formation was normal; the extracellular matrix was present, and the cells surface appeared smooth. While, Photo (2A) shows the biofilm of *C. albicans* treated with MIC value of 62.5mg/ ml after 48h. The extracellular materials were ruptured, with an inhibition in cells proliferation, and the cells had overall deformed morphology. Additionally, in Photo (3A), *C. albicans* biofilm formation treated with 62.5mg/ ml ULME for 72h showed an increase in the inhibition of cell proliferation and deformation in the morphology of cells compared to Photos (1A, 2A).

As illustrated in Photo (1B), *C. tropicalis* biofilm formation in the absence of ULME (control) after 48h showed the emergence of the microcolony formation extra polymeric material, and cells surface appeared smooth. While, Photo (2B) displays *C. tropicalis* biofilm formation treated with 62.5mg/ ml ULME for 48h exhibiting the followings: distortion of extracellular polymeric matrix; inhibition of cells proliferation; morphological deformation of cells' surface and rough cells. Additionally, Photo (3B) shows *C. tropicalis* biofilm formation treated with 62.5 mg/ml ULME for 72h. An increase was detected in the extracellular polymeric matrix distortion; inhibition of cells proliferation was recorded, cells' surface and rough cells were overall morphologically deformed, as compared to Photos (1B, 2B).

As shown in Photo (1C), *C. krusei* biofilm formation in the absence of ULME (control) after 48h reveals that, the extracellular matrix was present, and the cells surface appeared smooth. Whereas, the *C. krusei* biofilm treated with 62.5mg/ ml (Photo 2C) showed that, the extracellular polymeric matrix was distorted, aligned with an inhibition in cells proliferation, while cells' surface and rough cells were overall deformed morphologically. Moreover, Photo (3C) shows the biofilm of *C. krusei* treated with 62.5mg/ml ULME for 72h. There was an increase in the distortion of the extracellular polymeric matrix, an increasing inhibition in cells proliferation, and the cells surface and rough cells were overall morphologically deformed, compared to Photos (1C, 2C).

Finally, in Photo (1D), *C. glabrata* biofilm formation in the absence of ULME (control) after 48h monitored the presence of extracellular matrix and smooth appearance of the cells' surface. While, the *C. glabrata* biofilm treated with 62.5mg/ ml (Photo 2D) showed that the extracellular polymeric matrix was disturbed, number of cells was much lower, in addition to an inhibition of cells proliferation, while the cells' surface and rough cells were overall deformed morphologically. Photo (3D) represents the biofilm of *C. glabrata* treated with 62.5mg/ ml MEUL for 72h. An increase was noted in the distortion of the extracellular polymeric matrix; the number of cells was much lower, and increased cells' proliferation was recorded.

The examination of *C. albicans*, *C. tropicalis* and *C. krusei* is displayed in Photo (2A, B, C), respectively, without ULME (control). The normal *Candida* growth was noted with the formation of all the morphological structures of *C. albicans*, *C. tropicalis* and *C. krusei*, blastoconidia, pseudohypha and chlamydoconidia. Whereas, ULME at different concentrations of 1xMIC, 2xMIC and 4xMIC had an effect on the formation of the morphological structures of *C. albicans*, *C. tropicalis* and *C. krusei.* This effect is dependent on the concentration of ULME. The highest extract concentration inhibits the most morphological forms (blastoconidia, pseudohypha and chlamydoconidia), as shown in Photos (2, 3, 4A, B & C) separately.

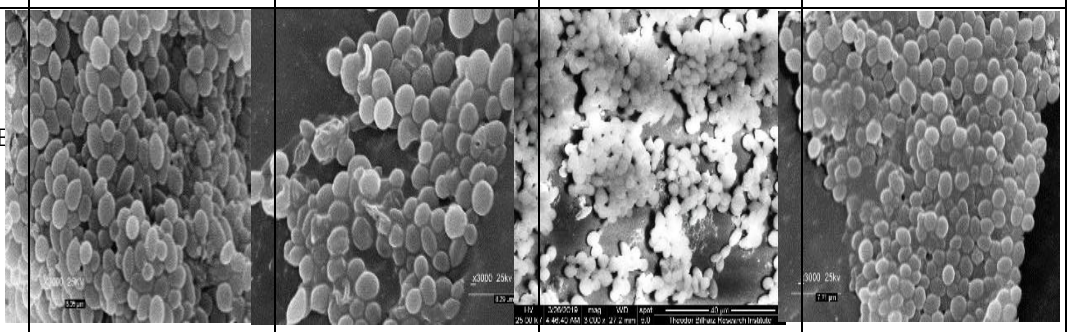
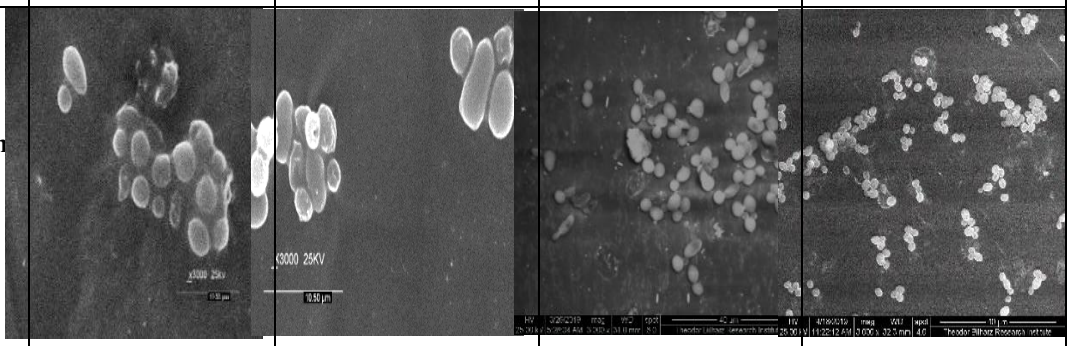
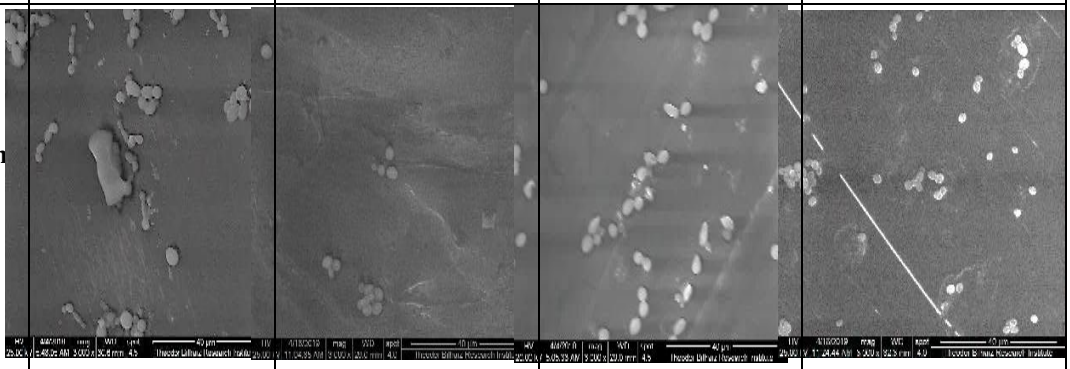
| | **Group A** *C. albicans* | **Group B** *C. tropicalis* | **Group C** *C. krusei* | **Group D** *C. glabrata* |
|---|---|---|---|---|
| **1- Not treated with ULME after 48h as control** |  | | | |
| **2-Treated with 62.5 mg/m of ULME after 48h** |  | | | |
| **3-Treated with 62.5 mg/m of ULME after 72h** |  | | | |

**Photo 1.** SEM photos showing the effect of ULME on *Candida* spp. biofilm formation at 3000x magnification

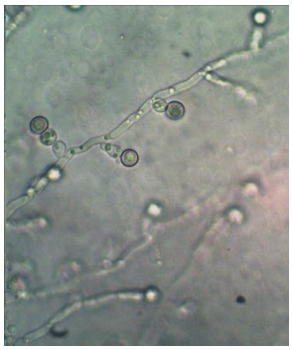
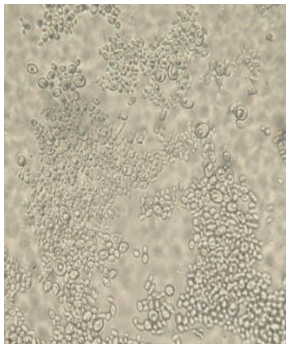
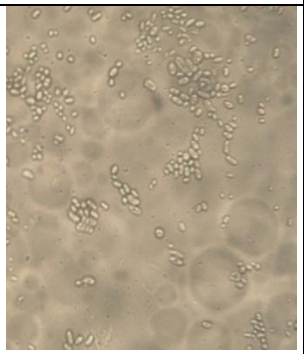
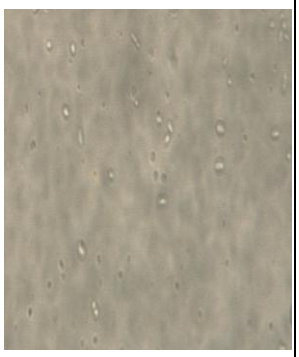
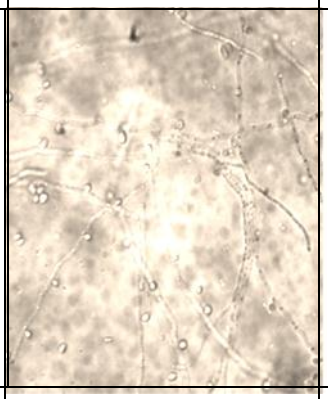
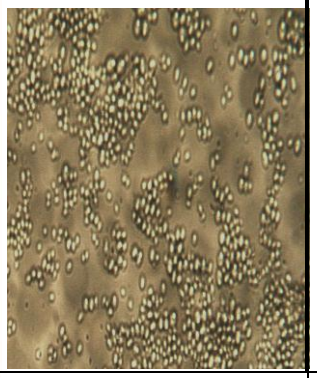
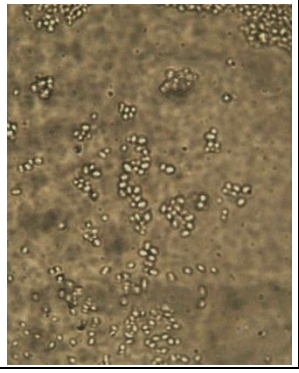
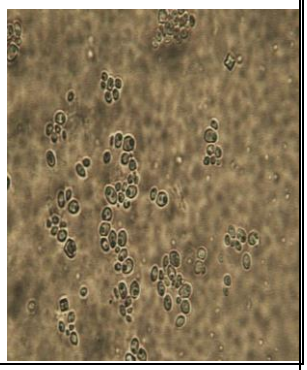
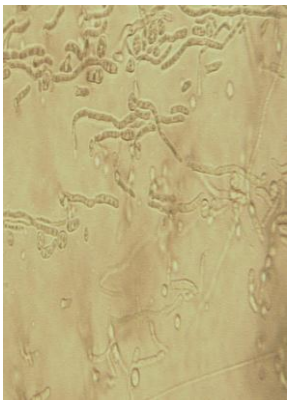
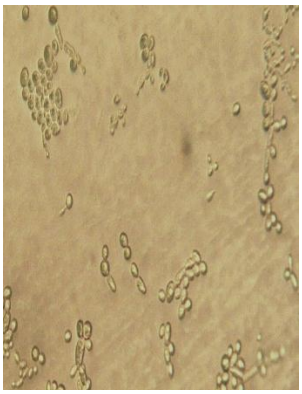
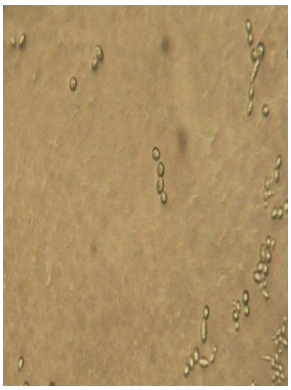
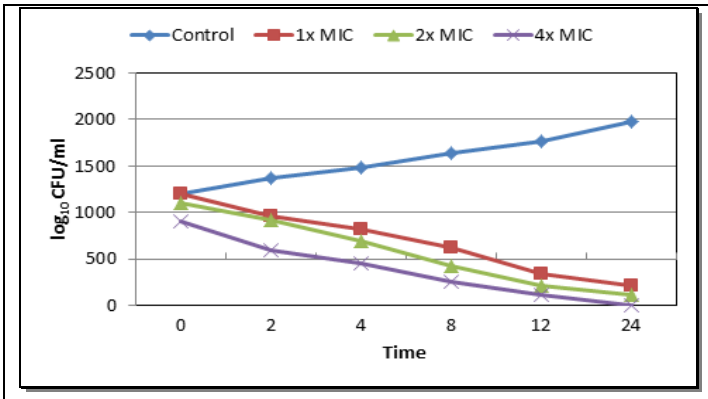
| | **1- Control** | **2- 1xMIC** | **3- 2xMIC** | **4- 4xMIC** |
|---|---|---|---|---|
| **A- *C. albicans*** |  |  |  |  |
| **B- *C. tropicals*** |  |  |  |  |
| **C- *C. krusei*** |  |  |  |  |

**Photo 2.** Effect of ULME on the micromorphology of *Candida* spp. (Magnification is 40X).

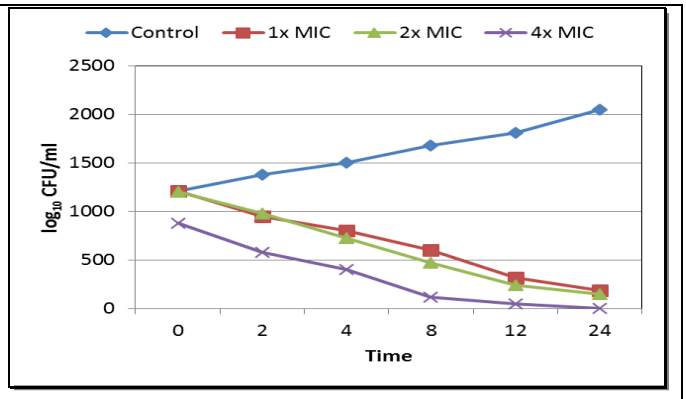
The tested *Candida* spp. treated with ULME at 2xMIC and 4xMIC (8-12h) showed a dramatic decrease in the viable cell counts, as presented in Figs. (5, 6, 7 & 8) $\log_{10}$ CFU/ml. Whereas, a drop in the viable cell counts was less at 1xMIC, compared to 2xMIC and 4xMIC. Moreover, 4xMIC of ULME showed elimination of the tested oral *Candida* spp. at 24h. The time- kill assay against the tested oral *Candida* spp. demonstrated that ULME was effective and fungicidal. *Candida albicans* had highly

significant variation among incubation time and the concentrations at $P \leq 0.01$; in addition, the interaction between incubation time and concentrations showed significant variation at $P \leq 0.05$, while *C. tropicalis* recorded highly significant differences between incubation time and the concentrations. Furthermore, *C. krusei* and *C. glabrata* showed highly significant variation among the concentrations of ULME at $P \leq 0.01$. Besides, the interaction between time and time and concentrations showed significant differences at $P \leq 0.05$.



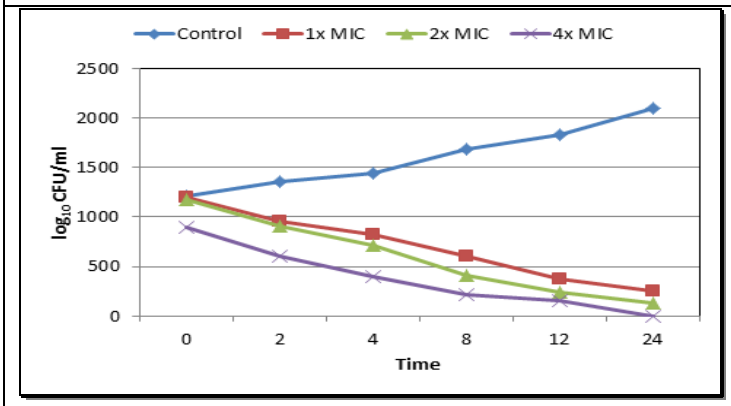

**F value for time → 5.25**, conc. →4.68**, time*conc. → 8.52*, *$P$<0.05, **$P$<0.01.**

**Fig. 5.** Time- kill curve of *C. albicans* plotted as the number of remaining viable cells (CFU/ml) against time



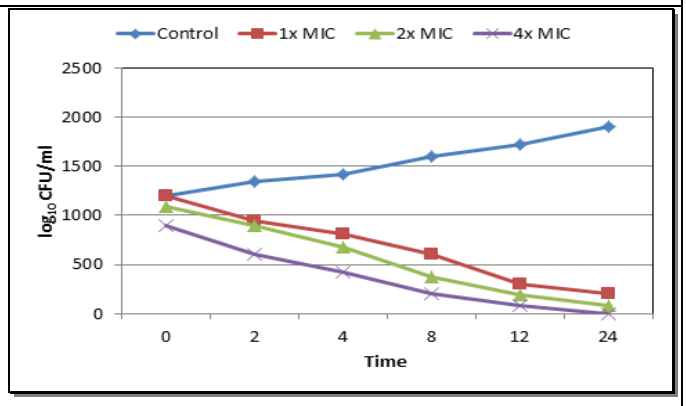

**F value for time → 4.48**, conc. →3.52*, time*conc. → 5.36*, *$P$<0.05, **$P$<0.01.**

**Fig. 6.** Time- kill curve of *C. tropicalis* plotted as the number of remaining viable cells (CFU/ml) against time




**F value for time →3.16*, conc.→5.52**, time*conc. →4.58*, *$P$<0.05, **$P$<0.01.**

**Fig. 7.** Time- kill curve of *C. krusei* plotted as the number of remaining viable cells (CFU/ml) against time




**F value for time→ 5.78*, conc. →5.11**, time*conc. → 4.58*, *$P$<0.05, **$P$<0.01.**

**Fig. 8.** Time- kill curve of *C. glabrata* plotted as the number of remaining viable cells (CFU/ml) against time

Cytotoxic effect of ULME on MRC-5 cell line viability is shown in Fig. (9); the results showed that ULME extract had no significant toxic effects against normal human lung

fibroblast cells in addition to exhibiting 50% inhibition $IC_{50}$ of MRC- 5 cell line at 1000 µg/ml.

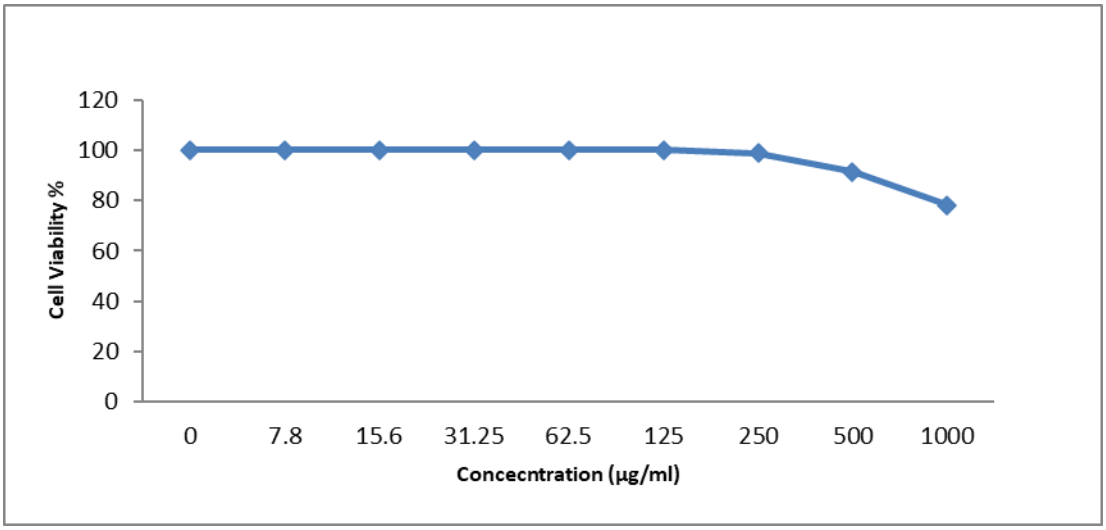



**Fig. 9.** Inhibitory activity of ULME against MRC-5 cell line

The GC/MS analysis of ULME revealed a mixture of diverse components. The overall number of key peaks was twelve; results indicated that the phytocompounds were observed and identified for ULME at 12 peaks as shown in Table (4) and Fig. (10). The primary chemical compounds found in high concentrations were palmitic acid (RT=11.63 min), followed by nonanoic acid (RT=12.69 min), 4-methoxyphenol (RT=11.28 min), Alpha-D-glactopyranose (RT=14.92 min), N-acetylglutamine, N,N', O,O'-tetrakis (RT=14.56 min), nonanoic acid (RT=7.4 min), d-galactose (RT=13.52 min), 11-Ocatdecenoic acid (RT=13.90), alpha-D-Glycophyranose (RT=12.30), 13-Octadecenoic acid (RT=13.30) and N-(4-chloro-phenyl (RT=14.52).

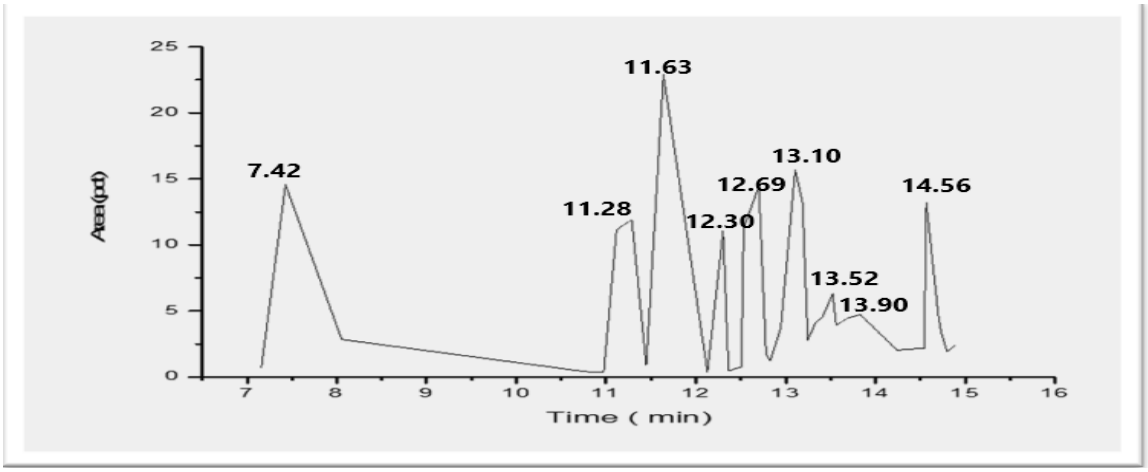



**Fig. 10.** GC/MS chromatogram of ULME

**Table 4.** GC/MS analysis for ULME major compounds

| Peak No. | RT (min) | Area (%) | Component name | Molecular formula | MW (mz) |
|---|---|---|---|---|---|
| 1 | 7.4 | 14.7219 | Nonanoic acid | $C_9H_{18}O_2$ | 158 |
| 2 | 11.28 | 11.566 | 4-Methoxyphenol | $C_7H_8O_2$ | 124 |
| 3 | 11.63 | 22.9294 | Palmitic acid | $C_{16}H_{32}O_2$ | 256 |
| 4 | 12.30 | 10.4223 | Alpha-D-Glucopyranose | $C_6H_{12}O_6$ | 180 |
| 5 | 12.69 | 14.9102 | Nonanoic acid | $C_9H_{18}O_2$ | 158 |
| 6 | 13.11 | 9.1621 | Nonanoic acid | $C_9H_{18}O_2$ | 158 |
| 7 | 13.30 | 3.0486 | 13-Octadecadienoic acid | $C_{18}H_{32}O_2$ | 280 |
| 8 | 13.52 | 5.9294 | d-Galactose, pentakis | $C_{22}H_{55}NO_6si_5$ | 570 |
| 9 | 13.90 | 4.3531 | 11-Octadecadienoic acid | $C_{18}H_{32}O_2$ | 280 |
| 10 | 14.48 | 2.3174 | N-(4-Chloro-phenyl | $C_{14}H_{12}CiNo$ | 245 |
| 11 | 14.56 | 13.5812 | N-Acetylglutamin | $C_7H_{12}N_2O_4$ | 188 |
| 12 | 14.92 | 3.1476 | Alpha-D-Glucopyranose | $C_6H_{12}O_6$ | 180 |

RT: Retention time MW: Molecular weight

## DISCUSSION

*Candida* infections are receiving more attention as a result of the increased use of broad-spectrum antibiotics and immunosuppressive agents; they are the leading cause of death and they pose a significant risk to hospitalized patients. As a result, the necessity of obtaining novel anticandidal compounds, especially from natural sources are very crucial. However, *Candida* infections have increased ten-fold **(Yenn *et al.,* 2012; Haque *et al.,* 2016)**. Secondary metabolites of marine algae with potential interest have been widely studied **(Tsang *et al.,* 2012)**. According to various investigations, antimicrobial activity is affected by algal species, extraction method, solvent type and the resistance of the studied organism **(El-Sheekh *et al.,* 2015)**. In the present study, methanol was the most effective solvent for the extraction of the bioactive compounds, followed by ethanol and chloroform. Whereas, hot and cold-water extracts had no effect against any of the tested oral *Candida* spp. ULME was the most effective against all the tested oral *Candida* species. These results are in accordance with many earlier reports, such as those of **Kandhasamy and Arunachalam (2008)**, **Osman *et al.* (2013)**, **Selim *et al*. (2015)** and **Sheikh *et al.* (2018)**.

In contrast, **Raj *et al*. (2017)** revealed that ethyl acetate extracts of *U. lactuca* showed the most effective solvent. Moreover, results revealed that the organic solvent provides a high efficiency in extracting compounds for anticandidal activities compared to water- based methods, which agree with the findings of **Axelsson and Gentili (2014)**. This could be due to the solvent's composition, which could improve the extraction of bioactive components from algal samples **(Sameeh *et al.*, 2016)**. Methanol is a good solvent for extraction, and it is frequently used in biology due to its high polarity. It may extract both lipophilic and hydrophilic molecules or substances. Another advantage is that, it can be easily removed at room temperature since it is highly volatile. Additionally, it can be very specific and broad, depending on the method of extraction, the organisms, the season of algal collection, the algal growth stages, experimental methods and interspecific variability in secondary metabolite production that may be related to seasonal variations. These differences could also be attributed to extraction protocols used to recover active metabolites and assay methods **(Karthikaidevi *et al*., 2009)**. Moreover, **Selim *et al*. (2015)** and **Sheikh *et al.* (2018)** found that the Chlorophyta had the strongest antimycotic effect.

The MIC and MFC of ULME against all the tested oral *Candida* spp. were 62.5 and 125mg/ml, respectively. In general, MFC values were greater than (but not equivalent to) MIC values in all treatments. **Siddiqui *et al*. (2013)** explaining the ratio of MFC/MIC determined that, if the substance had MFC/MIC ≥4, then it has a fungistatic activity, while if the substance had MFC/MIC < 4, it owns a fungicidal activity. According to our findings, the MFC of the tested extracts recorded higher concentrations, compared to the MIC assays. All extracts exhibited fungicidal properties that support their importance for the development of novel anticandidal agents, except for case of ethanol extract with *C. krusei* and *C. glabrata*. This fungicidal effectiveness could be due to the interfering with fungal protein production, DNA replication, or other cellular metabolism of the organism **(Pandima *et al*., 2010)**.

Bioactive molecules derived from marine algae have the ability to limit the growth of numerous pathogenic organisms as well as their biofilm metabolic activity **(El-Sheekh *et al*., 2015)**. Biofilm formation is considered the most important virulence factors of *Candida* species **(Kavanaugh *et al*., 2014)**. According to the findings of this investigation, secondary metabolites of ULME have the ability to reduce these virulence factors. *In vitro*, ULME decreased the metabolic activity of developed *Candida* species biofilms and functions as a major antibiofilm agent, preventing biofilm development and removing existing biofilm. These results agreed with **Mubarak *et al.* (2018)** who reported that seaweeds have antifungal and antibiofilm activities.

Scanning electron microscope images revealed morphological alteration of the tested *Candida* spp. The treated *Candida* spp. showed rough, irregular shape, with swelling, cell bursting and substantially less density, as compared to the control. These findings are considered promising for inhibiting biofilm formation of tested *Candida* species after 48

and 72 hours exposure to ULME, compared to the positive control "Fluconazole". This is probably caused by the presence of the bioactive compound in the crude extract. Cell death was caused by these cells morphological alterations, as previously documented for sophorolipid therapy of *C. albicans* **(Haque *et al.*, 2016)**. Many biosurfactants have been demonstrated to exhibit antimicrobial action via cell deformation and membrane distortion **(Gudiña *et al.*, 2013)**. According to **Sampaio *et al.* (2016)**, the composition of a substance's bioactive ingredient substantially influences its antifungal activity. At least, three parameters influence the efficiency of seaweed extracts in suppressing fungal growth: concentration, exposure time, and contact surface media **(Kenakin, 2006)**. There are no research papers demonstrating the role of ULME in *Candida* spp. biofilm formation. The discrepancies in anticandidal effects of the algal extracts could be attributed to changes in the active chemicals contained in the algae after extraction with different solvents. **(El Zawawy *et al.*, 2020)**. *Ulva lactuca* methanol crude extract altered the morphology of strains. Similar findings of **Sousa *et al.* (2016)** and **Freitas *et al.* (2017)** discovered that these extracts inhibit the micromorphology of *Candida* species, implying that they may have potential pharmacological activity as modulators of fungal biology. **Alves *et al.* (2013)** and **Leite *et al.* (2014)** explained that, specific morphological changes were associated with the pathogenicity of microorganisms, as local environmental factors that can alter the status of commensal fungi therapy making them infectious. The yeast-hyphal morphological transition is relevant to the virulence of fungal infections. The quantity and quality of the active compounds depend on the algal species, algal tissue and environmental factors. Therefore, further purification of the active compound and its antimicrobial activity from ULME may be recommended.

Therefore, the time- kill assay for the tested oral *Candida* spp. treated with ULME showed a decrease in the viable cell counts. **Freire *et al.* (2017)** who examined yeast growth as a function of time in the presence of various citral concentration discovered a reduction in the clinical *Candida* strain's growth. Moreover, **Zore *et al.* (2011)** observed the fungicidal effect of citral on *Candida albicans* using microbial death kinetic analysis. Time- kills curves confirmed the potency of ULME as an anti-candidal agent against the tested oral *Candida* spp. However, ULME has no significant toxic effects against normal human lung fibroblast cells. **Rodeiro *et al.* (2015)** found that, no significant cytotoxic effects were observed after exposing cells to *Ulva fasciata* extract. **Le *et al.* (2019)** also found that in MTT assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium, cell viability was not significantly affected by *Ulva pertusa* at concentrations less than 200 µg/ml when compared to untreated cells; but at 400 µg/ml of *Ulva pertusa*, cell viability reduced significantly ($P< 0.05$). While, *Ulva pertusa* had no cytotoxic effects at concentration ranging from 50 to 200µg/ ml. The present findings showed that MTT assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium of ULME did not significantly affect cell viability of normal MRC-5, with inhibition concentration of $IC_{50}$=>1000 µg/ml. ULME had no cytotoxic effects and is safe to use.

The GC-MS chromatograms showed the compounds that are present in ULME. The major component with eleven peaks indicates twelve compounds. In the study of **Selim *et al*. (2015)**, it was elucidated that, the major compound of GC-MS analysis for methanolic extract of *Ulva lactuca* has 12 peaks, indicating the presence of 12 compounds. The most abundant saturated fatty acid is the palmitic acid (C16:0) (22.92%), which is similar to the finding of **Caf *et al.*, (2015)** who found that the green algae have high concentration of palmitic acid (C16:0) (41.68%). According to the GC-MS analysis, the chemical composition of the most promising ULME contained four significant peaks; palmitic acid was the principal component with the largest peak area percentage when compared to the other components. In addition, this compound was previously detected by GC-MS obtained in the study of **Shobeir *et al.* (2016)**. ULME contains major components, palmitic acid, trichloromethyloxirane, linolenic acid, ethylester, 3,7,11,15-tetramethyl – 2 – hexadecen – 1-ol, 11-octadecenoic acid, 12,15-octadecadienoic acid and methylester. The majority of the discovered components have been documented to exhibit antimicrobial action, which could be responsible for the antifungal activity. These results are in close agreement with the findings of **Thibane *et al*. (2010)** who found that, marine polyunsaturated fatty acids influenced the human pathogenic *C. albicans* and *C. dubliniensis*. The third major component observed was 4-Methoxyphenol; in this context, **Sujina *et al.* (2016)** stated that, Phenol, 2-methoxy-4-(2-propenyl) that was previously detected by GC-MS analysis of ULME had high antimicrobial and antioxidant activities. Marine macroalgae are the only organisms that have the enzymes required to produce long-chain polyunsaturated fatty acids (PUFA). **Kumari *et al.* (2011**) discovered that *U. lactuca* is a green alga rich in C20 PUFAs.

The effect of ULME on *Candida* spp. biofilm formation affected the structure of *Candida* spp. which might be due to marine bioactive compounds including fatty acids and phenols affecting the cellular morphology of biofilms, as well as increased oxidative stress because of the incorporation of the fatty acids into the cellular lipids. These findings imply that marine fatty acids could be effective in the treatment and/or prevention of *Candida* biofilms that have higher antifungal resistance than free-living cells. These findings show that ULME is a mixture of multiple chemicals, and that each component may contribute more to biofilm inhibition than if they worked alone.

## CONCLUSION

The present findings proved that ULME may be used as a natural anticandidal agent in known dosages. It contains anticandidal metabolites that could be used to treat oral candidiasis infections because they can inhibit *Candida* growth by reducing biofilm formation, hyphal growth and adhesion genes. However, conventional drugs are unaffordable or unavailable and health facilities inaccessible particularly in developing countries. ULME may be an alternative to the conventional antifungal drugs for the

treatment of oral candidiasis. However, additional research is needed to investigate and explore the mechanism of the action of this extract in addition to the efficacy of each component of the extract by itself on *Candida* spp. biofilm formation.

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