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Evaluation of Antifungal Activity of some Plant Extracts Against Causing Agents of Water-Related Fungal Keratitis

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HIGH levels of pollutants in the River Nile water caused severe alteration in the pollutant indicators such as temperature, pH, dissolved oxygen, chemical oxygen demand, ammonia and nitrate; in addition to the concentration of some heavy metals (*e.g.* lead, mercury and cadmium) which made water unsuitable for drinking, irrigation and aquatic life. Microbiological results indicated that Rosetta branch had subjected to sewage pollution with the common occurrence of *Candida pelliculosa, Candida tropicalis, Trichosporon mucoides* and *Aspergiulls niger*. The infectious mycotic keratitis among patients of the surrounding residential area was closely related to the identified yeasts and filamentous fungal isolates from water samples. Methanolic leaf extract of *Melaleuca alternifolia* tree showed a significant inhibition for the most common yeast and filamentous fungal causative agents, especially against *Candida pelliculosa* and *Trichosporon mucoides*, confirmed with ultra-structural cellular distortions, that were observed by transmission electron microscope (TEM). The minimum inhibitory concentrations (MIC) were also recorded. *Melaleuca alternifolia* leaf extract was analyzed by Fourier transformation Infra-red (FT-IR) spectroscopy and gas chromatography / mass spectrum (GC/MS).

Keywords: River Nile, Pollutant Indicators, Water-Related Infectious Diseases, *Candida pelliculosa*, *Trichosporon mucoides, Melaleuca alternifolia*

River Nile is the main source of water for drinking, agriculture, industry, navigation, recreation and fish production in Egypt. Thus, this River is of dominating influence on the economic, cultural, public health, social and political aspects of the country (Rabeh, 2007). Concerning the pollution of Rosetta Branch, there are three main sources which potentially affect and deteriorate its water quality. El-Rahawy Drain pours more than $400x10^3$ m^3 daily

including 398 x$10^3$$m^3$ liquid sewage and 2 x10^3 m^3 of sludge from Giza Governorate (Ghallab, 2000). At the same time, Sobol Drain at Menofyia Governorate pours its agricultural wastes directly into water current. The second source is Kafr El-Zayat Industrial Area, which includes the industrial effluents from the factories of super-phosphate and sulfur-compounds (*e.g.* El-Malia Company), oil and soap industries (*e.g.* El-Malh and Soda Company) and pesticides factory, which pour their effluents directly into water current without any treatment. The third source is many agricultural drains, to which the sewage discharged from many cities and villages that are distributed along the river banks (Abdo, 2002). Water quality of Rosetta Branch may be changed by several factors in the last decades. So, it is important to study the physicochemical characteristics of both water and sediment in the Rosetta Branch (El-Amier *et al.*, 2015a).

Fungi are heterotrophic worldwide spreading microorganisms commonly inhabit many environments in contact with human beings. Certain fungi can inhabit normally the human body surface, which are called human normal microflora. Some fungi inherit, or acquire pathogenic characters and can attack the intact human tissues; while other opportunistic fungi can invade the injured human skin (Shukla and Singh, 1997). Human mycosis is a disease caused by fungi, causing inflammations, and many tissue disorders in different sites within the human body, and thus becoming an important constituent of worldwide public health problems (Rodrick, 2000). Human mycosis can be divided into five categories according to the site of infection and attacked tissues. The first group is the superficial mycosis, growing on the outer surface of skin (*e.g.* tinea diseases) and mucous membranes (*e.g.* candidiasis in children). Secondly, cutaneous infections, which can be established within outer layer of skin, decaying and feeding on keratin of nails and hairs. Skin infections are mostly caused by a physiologically distinct group of fungi called dermatophytes, including different species of *Microsporum*, *Trichophyton* and *Epidermophyton*. In addition, third group includes the subcutaneous infections, which invade the living layers of skin, and cause more severe inflammations for living tissues. More deep infections are grouped in the forth category, which cause systemic fungal diseases in different body organs by spore dissemination through the blood stream, and other body fluids (i.e. aspergillosis in lung and cryptococcosis in central nervous system); the fifth group of fungal infections can be separately named ocular mycosis, invading the human eye (Keay *et al.*, 2006).

Mycotic keratitis is a disease caused by fungal invasion into the corneal stroma. It is an exogenous infection, attacking the injured corneal epithelium and establishes the fungal growth within the collagen bound lamellae of stromal layer of cornea. Mycotic keratitis is considered to be a resistant corneal ulcer, which possesses aggressive microbial growth, and becomes not responding to conventional treatments for at least one week or more and even becomes worse due to continued fungal growth (Tanure *et al.*, 2000). The World Health Organization (WHO) stated, in its Global Initiative Vision Report, that about 10 million cases all over the world

will become blind by the year 2020 due to different resistant corneal ulcers, including those of fungal origin (Gorden, 1999).

Plant extracts can be used as medicinal components that can inhibit pathogenic growth. Studies of plant extracts have been conducted to evaluate the characteristics of natural drug products, including their sustainability, affordability, and antimicrobial activity. A considerable number of studies on medicinal plants and alternative compounds, such as secondary metabolites, phenolic compounds, essential oils and extracts, have been performed (Negri *et al.*, 2014).

Therefore, the present study aimed to evaluate the contamination level of River Nile (Rosetta branch) at Kafr El-Zayat industrial area. Many parameters were estimated, mainly the microbial contamination, for evaluating the relationship between the spread of fungal populations in this main water resource and the incidence of different eye infectious diseases among patients inhabiting the surrounding area. Extracts of ornamental and street trees would be explored as a source of antifungal compounds in a trial to offer a cheap and effective treatment for widely spread fungal infections along River Nile.

Materials and Methods

Study Area

Sampling process was carried out in winter season (Febraury 2013). Three stations were selected for the collection of water samples (Fig. 1 and 2), sampling point 1 at the front of Kafr Hashad village (before Tala drainage and industrial region drains); sampling point 2 in a common site after the effluent of El-Malia Company (fertilizer producing company which its industrial effluents contain super-phosphate and sulfur-compounds) and El-Malh and Soda Company (oil and soap company which its industrial effluents contain surfactants, oil and soap residues) at Kafr El-Zayat city; and sampling point 3 at the entrance site of Benover village water treatment station.

Water Analysis

Water samples were collected at about 1m depth from the three points, using sterilized plastic bottles. The collected samples were stored, refrigerated and analyzed immediately after arrival to the laboratory. For analysis of heavy metals, samples were preserved immediately after collection by acidifying with concentrated HNO_3 to pH < 2 using 5 ml nitric acid for 1 liter sample. After that, samples were stored in a refrigerator at 4°C to prevent change in volume due to evaporation. Samples might be filtered, if necessary, and a portion of the filtered sample was taken for the required determination. Air and water temperatures (°C), electrical conductivity (µS cm^{-1}) and hydrogen ion concentration (pH value) were measured directly at the sampling stations using portable water quality checker (U-10 HORIBA). Dissolved oxygen was carried out using the modified Winkler method, Ammonia using Nesslerization method,

Nitrate-Nitrogen using ultraviolet spectrophotometric screening method, and some heavy metals (Lead, Mercury and Cadmium) using nitric acid digestion method (APHA, 2011).

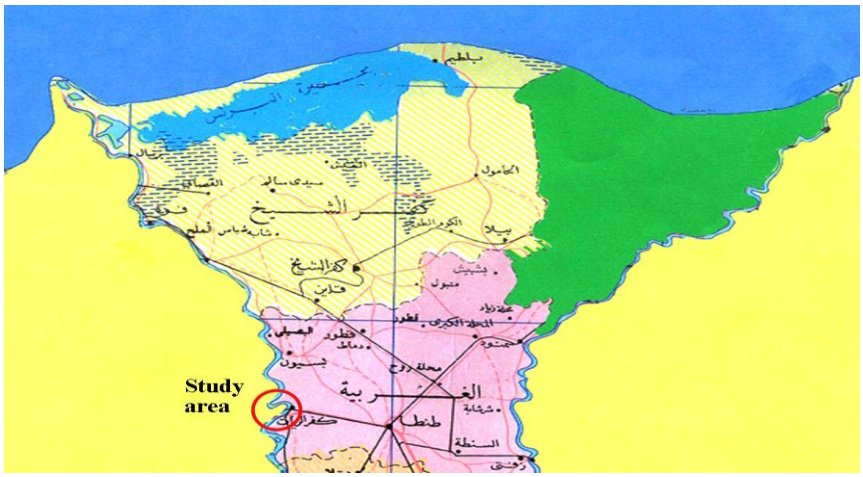


Fig. 1. Map of Nile Delta, showing the study area (Rosetta branch of River Nile at Kafr El-Zayat industrial area).

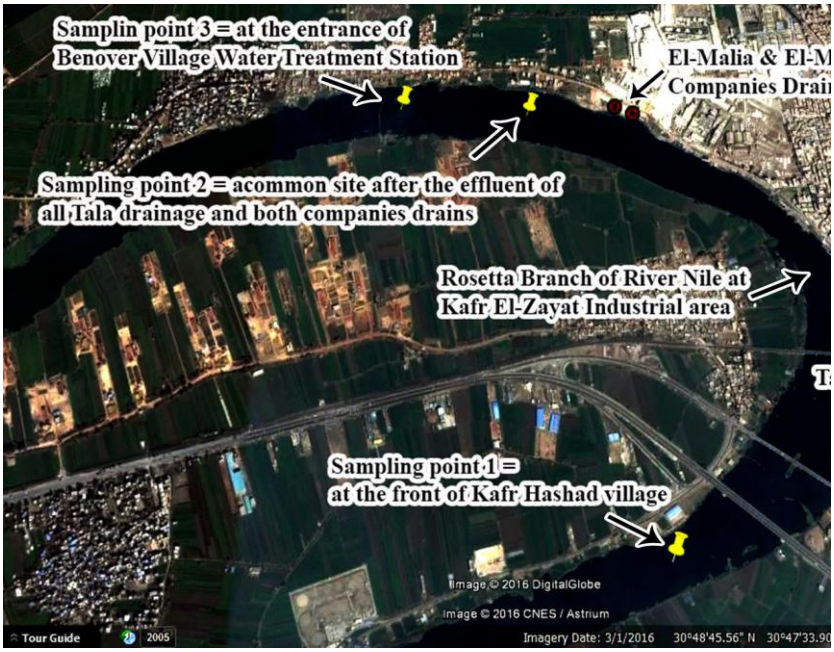


Fig. 2. Representative view for the three points of water sample collection, google earth.

Microbiological Analysis of Water Current and Keratitis Patients

Sterile 100 ml plastic bottles were used to collect water samples. Surface plate method (APHA, 2011) was used for determination of the total bacterial count at 37 °C for 48 hr using Plate Count Agar medium. The plate count technique was used for the isolation and enumeration of fungi (Dutka, 1989), using Aureomycin Rose Bengal Glucose Peptone Agar (ARGPA). Aliquot of 0.1 to 1 ml of water sample was plated out, and left to be solidified. After solidification, Petri dishes were inverted and divided into two groups, one was incubated at 27 °C for 3 days and the other was incubated at 37 °C for 48 hr, then the number of colonies were counted on each plate and recorded as $CFUml^{-1}$. After enumeration, fungal colonies were transferred to Sabouraud's Dextrose Agar plates for identification purposes.

Fungal isolates were collected from infected human eyes during regular once a week visits to the outpatient clinic, and daily follow up visits for patients admitted to Inpatient Department of Ophthalmology Hospital, Tanta University, Egypt. Samples were collected from 30 patients that were clinically diagnosed by the physician's team of the clinic to possess mycotic corneal ulcers. All samples were collected by flamed "Kumara" platinum spatula (Alcon-Couvreur, Belgium) for hard tissues, or by Ethylene gas-sterilized single use cotton swab (BioMed, China) for soft ulcers to avoid perforation. Aseptic conditions of sampling were considered by 1- avoiding conjunctiva and eye lids during corneal scraping; 2- Betadine (Nile Pharma, Cairo, Egypt.) was applied as a gentle washing around infected eyes; 3- samples were isolated in a closed controlled room in the hospital laboratory. Then C-streaks were made on culture plates to distinguish microbial growth of corneal scraping from other plate contaminants (Margo and Brinser, 1987). Each sample was cultured on 2 sterile Petri dishes with sterile Sabouraud's dextrose agar (SDA) medium, One plate was incubated at 27°C for 3 days, and the other plate was incubated at 37°C for 48hr. Fungal colonies were subcultured for purification, and identification on the same medium under the same incubation conditions.

Another group of fungal cultures was used as archive confirmation for the present survey. Some cultures were previously collected from water samples at the same study area during 2005-2006, identified in Mycology Research Unit, Botany Department, Faculty of Science, Tanta University; and the other cultures were previously isolated from mycotic keratitis, followed up in the Ophthalmology Hospital, Tanta University from patients of the surrounding residential area of our study, represented to in the Ocular Microbiology Unit, Ophthalmology Hospital, Tanta University.

Isolated filamentous fungi were identified morphologically by examination under light microscope at 400X. The identification process was according to Barnett (1972) for the genera of fungi imperfection, Barron (1968) for the genera of Hyphomycetes, Domsch *et al*. (1980) for compenedium of soil fungi, Ramirez (1982) for *Penicillia* and Raper and Fennell (1965) for *Aspergillus* species. The plate count technique was used for the isolation and enumeration

of yeast (Dutka, 1989). Suspected colonies of yeast were streaked onto Sabouraud dextrose agar plates, and incubated for 48 hr at 37°C. These cultures were used as the source of inoculum for the yeast confirmatory tests. The isolated yeasts were identified according to Lârone (1995) using API 20 CAUX from Biome´rieux SA (France).

Antifungal, Antioxidant Activity and Phytochemical Screening of the Collected Plant Extracts

Leaves of 14 cultivated ornamental and street trees were collected during spring, 2014 from Tanta city, Egypt (30.80° N, 30.99° E), as shown in Table 1. Some representative plant samples were identified and deposited in the herbarium of Botany Department, Faculty of Science, Tanta University, Egypt (CTANE). Plant materials were air dried and ground to fine powders by electrical mixer. Ten grams of each leaf sample were placed in the Sohxlete and extracted successively with 100 ml of methanol for 48hr. The extracts were evaporated to dryness under reduced pressure using rotatory evaporator at 35°C, weighed, then stored at -80°C. At the experimental time, these extracts were re-dissolved in 10 ml aqueous methanol (70%).

TABLE 1. List of selected plants used for antifungal activity screening.

Latin name	**English name**	**Family**	**Arabic name**
Delonix regia Hook	Royal Poinciana	Fabaceae	بوانسيانا
Casuarina equisetifola L.	Horsetail tree	Casuarinaceae	جازورينا
Kigelia africana (Lam.) Benth.	Sausage tree	Bignoniaceae	كيجيليا
Ficus sycomorus L.	Sycamore fig	Moraceae	جميز
Araucaria heterophylla (Salisb.) Franco	Norfolk Island pine	Araucariaceae	اروكاريا ابرية
Tamarix aphylla (L.) Karst.	Athel tamarisk	Tamaricaceae	اثل
Ficus benjamina L.	Benjamin's fig	Moraceae	فيكس
Cassia nodosa L.	Pink Cassia	Fabaceae	كاسيا نودوزا
Melaleuca alternifolia (Maiden & Betche) Cheel	Narrow-leaved tea-tree	Myrtaceae	ميلالوكا
Dalbergia sissoo Roxb.	North Indian rosewood	Fabaceae	سرسوع
Azadirachta indica A.Juss.	Neem	Meliaceae	نيم
Bougainvillea glabra Choisy	Paper flower	Nyctaginaceae	جهنمية
Magnolia grandiflora L.	Southern magnolia	Magnoliaceae	ماجنوليا
Callistemon viminalis (Sol. ex Gaertn.) G.Don	Weeping bottlebrush	Myrtaceae	فرشاة الزجاج

Cut plug method was employed to determine the antifungal activity of tested extracts (Pridham *et al.*, 1956). The best extracts that showed antifungal activities later were tested to determine the minimal inhibitory concentration against the tested fungi. Serial dilutions were made for selected extracts in order to prepare concentrations of 25, 50, 100, 200 and 300 mg ml^{-1} of the plant methanolic extract (Shadomy *et al.*, 1985). For studying the effect of *Melaleuca alternifolia* methanolic leaf extract on the ultrastructure of *Trichosporon mucoides* and *Candida pelliculosa* cells, tested microorganism was cultured on the appropriate liquid nutrition medium to get a cell suspension of $5x10^6$ cells ml^{-1}, and then mixed with *Melaleuca alternifolia* methanolic leaf extract of the previously recorded MIC. All the mixture was incubated overnight on a shaking incubator of 60 rpm at the appropriate temperature. Then treated mixture was centrifuged at 3000 rpm for 20 min, washed with sterile saline solution, re-centrifuged to collect the cell pellet in a clean Eppendorf tube (Richards and Cavill, 1976). Sample was embedded in araldite 502 resin to build a plastic mold (for complete fixation of all cell contents), that were cut into semi-thin sections in the ultra-cut microtome (LEICA ultracut UCT, Japan), stained with 1% toleudine blue, examined to confirm the success of sample preparation, then ultra-thin sections were prepared, stained with uranyl acetate, and counter stained with lead citrate (Ardenne and Beischer, 1940). Full-stained ultra-thin sections were examined, and photographed under the appropriate magnification (at10000X for larger cells of fungi, or 20000X for smaller cells of bacteria), using the transmission electron microscope (JEOL-JEM-100SX, Japan) with beam current = 60µA, and high voltage of 80 KV and was applied in Electron Microscope Unit at Faculty of Medicine, Tanta University.

The antioxidant capacity of the selected plant extracts were investigated according to Brand-Williams *et al*. (1995) and Bondet *et al.* (1997), slightly modified as following: DPPH (2,2 Diphenyl -1-picrylhydrazyl) was shaken vigorously with the extracts and the decrease in absorbance of the resulting solution was monitored at 517 nm after 1 hr. Flavonoids were estimated by calibration curve plotted by quercetin as a standard flavonoid, using Aluminium chloride colorimetric technique (Chang *et al.*, 2002) and expressed as mg g^{-1}. Total phenolic content was estimated quantitavely using the method described by Jindal and Singh (1975) using Foline-Ciocalteu's reagent and Na_2CO_3 (20%). A standard curve by gallic acid was used for the determination of the total phenolic compounds content (mg g^{-1}). The methanolic leaf extract of *Melaleuca alternifolia* was subjected for the chemical analysis through gas chromatography / mass spectrum (GC-MS) analysis according to Freedman *et al*. (1986); Fourier transformation Infra-red (FT-IR) analysis (Shimadzu IR affinity 1, Japan) in the Central Lab of Tanta University.

Results

Evaluation of Water Physico-Chemical Characters

The present study showed that the mean water temperature recorded during winter (February, 2013) was (19.3 °C), while the mean electrical conductivity (EC) was 775 µS cm^{-1}. Sampling point 1 was characterized by the highest value of pH, dissolved oxygen (DO), chemical oxygen demand (COD), ammonia, nitrate and lead; sampling point 2 was characterized by the highest value of ammonia; whereas sampling point 3 was characterized by the highest value of temperature, conductivity, total dissolved solids (TDS) and cadmium (Table 2).

TABLE 2. Evaluation of the water physico-chemical characters (mean ± SD) of the River Nile at Kafr El-Zayat Industrial Area during winter (2013).

Character		Sampling point			Mean
		1	2	3	
Temperature (^{o}C)		19.2	19.2	19.6	19.3 ± 0.2
EC (µS cm^{-1})		745	786	794	775.0 ± 26.3
pH		7.63	7.57	7.57	7.6 ± 0.03
Total dissolved solids	(mg L^{-1})	395	417	421	411.0 ± 14.0
Dissolved Oxygen		3.5	2.6	2.8	2.9± 0.5
COD		33	30	30	31.0 ±1.7
Ammonia		8.5	8.5	8.25	8.4 ± 0.1
Nitrate		6.65	6.16	6.0	6.3 ± 0.3
Cd^{+2}	(ppm)	0.003	0.003	0.004	0.003 ± 0.001
Pb^{+2}		0.031	0.025	0.024	0.03 ± 0.004
Hg^{+2}		0.054	0.016	0.007	0.03 ± 0.02

Samplin point 1= at the front of Kafr Hashad village.
Samplin point 2= a common site after the effluent of all Tala drainage and both companies drains.
Samplin point 3= at the entrance of Benover village water treatment station.

Counts of Total Bacteria, Yeasts and Filamentous Fungi Counts

The highest values of TBCs (total bacterial counts) at 37°C were 7803 x10^{3}CFU/ml (Table 3), recorded at sampling point 1. Highest count of fungi (350 colonies /ml) at 27°C at station 2 was recorded (Table 3). Highest count of yeast (1480 colonies /ml) at 37°C was recorded at station 1 during the same season. The most abundant yeasts were *Trichosporon mucoides, Candida albicans, Candida pelliculosa* and *Candida tropicalis*, while the most abundant fungi were *Aspergillus terreus, Trichoderma viride* and *Aspergillus niger*. During present survey and previous archive collection yeasts and fungi isolated

from the infected eyes with mycotic keratitis were closely related to that isolated from the water samples (Table 4).

TABLE 3. Total bacterial, yeasts and fungal counts of the River Nile water at Kafr El-Zayat Industrial Area during winter (2013).

Station	Total Cell Count ($CFUml^{-1}$)		
	Bacteria ($37\ ^oC \times 10^3$)	Yeast ($37\ ^oC$)	Fungi ($27\ ^oC$)
1- Kafr Hashad village	7803	1480	20
2- Common industrial effluent	589	20	350
3- Benover village	151	650	100

TABLE 4. Distribution of yeast and fungal species in River Nile and infected eyes during the present survey and the previous archive collections.

Species	River Nile	Infected eye
Yeasts		
Rhodotorula glutinis Harrison, 1928	+	+
Candida albicans Berkhout, 1923	+	+
Candida ciferrii Kreger-Van, 1965	+	+
Candida pelliculosa Redaelli, 1925	+	+
Candida tropicalis Berkhout, 1923	+	+
Trichosporon mucoides Bhrend, 1890	+	+
Stephanoascus ciferrii Smith, 1976	+	+
Candida glabrata Anderson, 1917	+	-
Saccharomyces cerevisiae Hansen, 1875	+	-
Candida magnoliae Lodder & Kreger, 1952	+	-
Filamentous Fungi		
Cladosporium carrionii Link, 1816	+	+
Penicillium marneffei Segretain, 1959	+	+
Aspergillus terreus Thom, 1918	+	+
Aspergillus niger Tieghem, 1867	+	+
Aspergillus tamari Micheli, 1729	+	-
Trichoderma viride Pers., 1794	+	-
Total	16	11

+ = Present - = Absent

Predisposing Factors of Mycotic Keratitis:

Table 5 revealed that mycotic ulcers were observed to be common among patients aged more than 40 years old (18 cases=60% out of total mycotic ulcers), and lowered gradually among youth and children to be only 2 cases (6.7%) for patients of less than 20 years old. It was noticeable that mycotic keratitis was more common in males (16 cases= 53.3%) more than females (14 cases= 46.7%). Patients holding rural houses possessed higher incidence of mycotic ulcers (19 cases= 63.3%), more than those in urban houses (11 cases= 36.7%), that was confirmed by the higher incidence of mycotic ulcers (24 cases = 80%) among patients having

indoor pipes water source than outdoor pumps (6 cases= 20%). Examination of the infected eye showed higher incidence of mycotic infection in the left eye (18 cases=60%) than in the right eye (12 cases= 40%).

TABLE 5. Incidence of mycotic ulcers in relation to patient's age and other human life conditions during the present survey.

Parameter	Category	No of cases	Percentage (%)
Age	<20 year	2	6.7
	20-40 year	10	33.3
	>40 year	18	60
Gender	Male	16	53.3
	Female	14	46.7
Type of house	Rural	19	63.3
	Urban	11	36.7
Water source	Indoor pipes	24	80
	Outdoor pumps	6	20
Infected eye	Right	12	40
	Left	18	60
Total		30	100

Antifungal Activity of Plant Extracts

The methanolic extracts of the selected plants were screened for inhibitory efficiency against *Candida pelliculosa*, *Candida tropicalis*, *Trichosporon mucoides* and *Aspergillus niger*, the most effective methanolic plant extract was *Melaleuca alternifolia* leaf extract, which gave 19 mm inhibition zone (Table 6). The minimal inhibitory concentration of *Melaleuca alternifolia* leaf methanolic extract was recorded at 100 mg ml^{-1} against *Trichosporon mucoides*, but 200 mg ml^{-1} against *Candida pelliculosa* and *Candida tropicalis* isolates (Fig. 3).

TABLE 6. General survey for antifungal activity of the tested plant extracts against isolated yeast.

Plant	Mean of inhibition Zone (mm)		
	Candida pelliculosa	***Candida tropicalis***	***Trichosporon mucoides***
Delonix regia	12±0.06	0.00	12±0.06
Casuarina equisetifola	19±0.06	0.00	17±0.06
Ficus sycomorus	0.00	0.00	12±0.00
Tamarix aphylla	11±0.06	11±0.06	11±0.00
Melaleuca alternifolia	19±0.00	19±0.00	19±0.06
Azadirachta indica	15±0.06	0.00	16±0.06
Callistemon viminalis	12±0.06	0.00	13±0.06
Positive control (Fluconazole 20 mg ml^{-1})	20±0.00	20±0.00	20±0.00

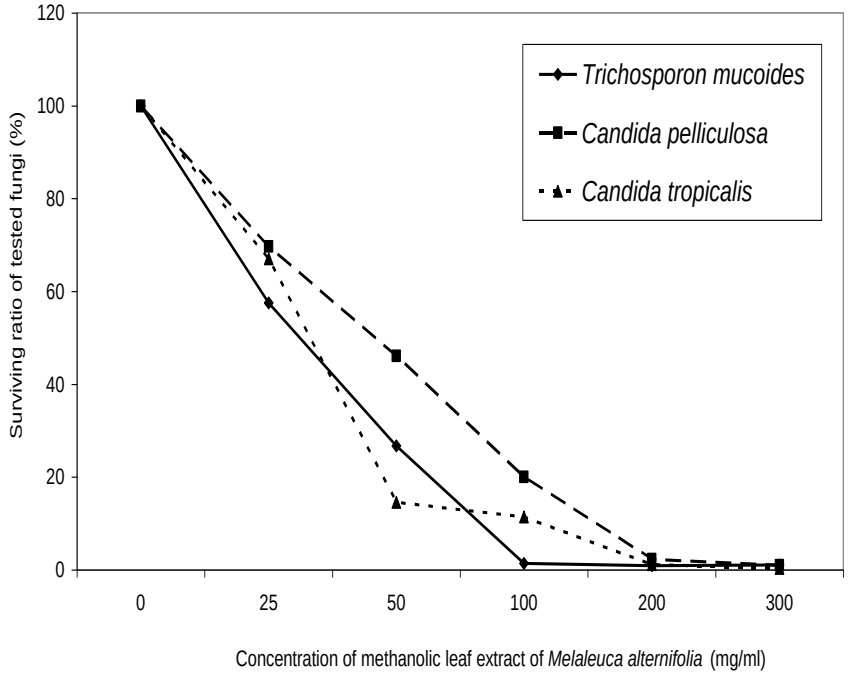
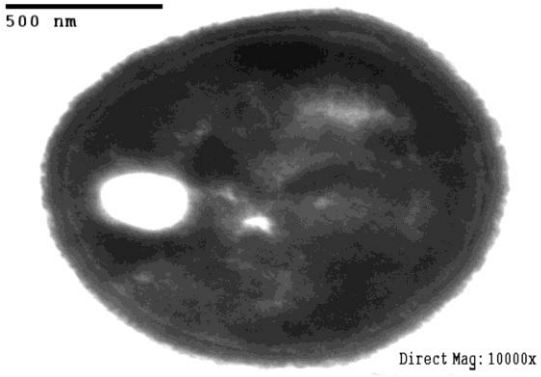


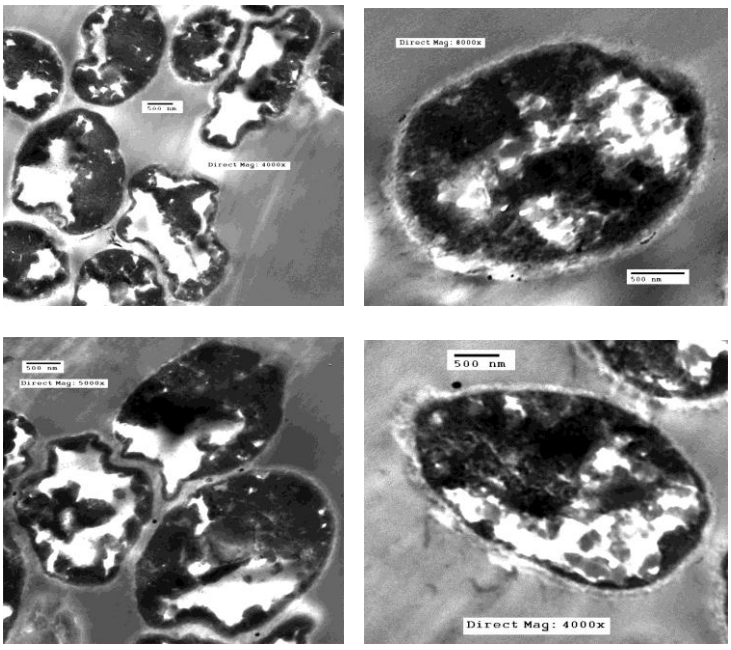
Fig.3. Surviving ratio of the isolated fungal pathogens against *Melaleuca alternifolia* leaf methanolic extract.

Ultrastructural Examination of Candida pelliculosa and Trichosporon mucoides Treated with Melaleuca alternifolia Methanolic Leaf Extract

Effect of *Melaleuca alternifolia* leaf methanolic extract on the cell wall and structure of *Candida pelliculosa* and *Trichosporon mucoides* under transmission electron microscope were showed in Photos 1 and 2, respectively. Transmission electron micrographs of non-treated (control) cells, showed intact cell wall and cytoplasmic contents, without any foreign precipitations inside, or outside the cell circumstance. Treated cells of *Candida pelliculosa* showed observable agglutination of nuclear material, lysis of nuclear membrane and dissolving of cellular lipids. Also, rupture of cell wall, perforation of cell membrane and leakage of cell nutrients and soluble contents were recorded. Treated cells of *Trichosporon mucoides* showed severe destruction of cell wall, agglutination of cell proteins and deformation of cell shape due to leakage of cytoplasmic soluble contents and water. These effects were referred to the antimicrobial activity of *Melaleuca alternifolia* methanolic leaf extract, where it acted as a protein coagulant, lipid solvent and dehydrating agent due to its high content of phenolic compounds and the presence of other biologically active fatty acids and flavonoids.

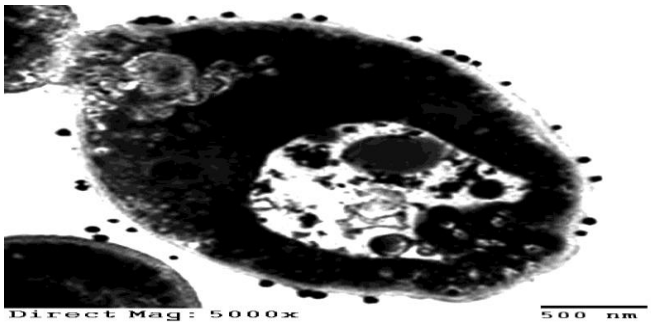


Control *Candida pelliculosa* cells.

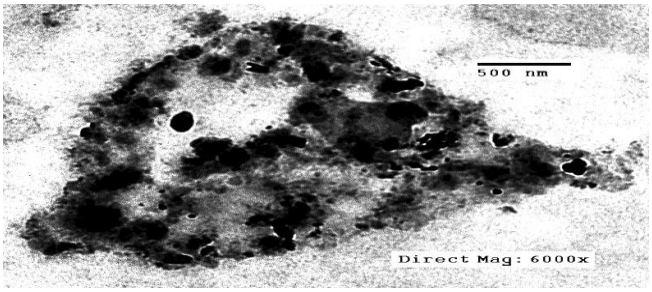


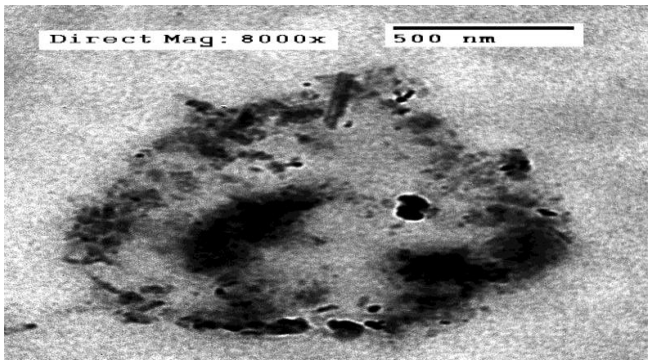
Treated *Candida pelliculosa* cells.

Photo. 1. Transmission electron micrograph (TEM) of *Candida pelliculosa* treated with 200 mg ml^{-1} of *Melaleuca alternifolia* methanolic leaf extract, compared with control.



Control *Trichosporon mucoides* cells.





Treated *Trichosporon mucoides* cells.

Photo . 2. Transmission electron micrograph (TEM) of *Trichosporon mucoides* treated with 100 mg ml^{-1} of *Melaleuca alternifolia* methanolic leaf extract, compared with control.

Analysis of Methanolic Leaf Extract of the Selected Plants

Quantitative analysis of the methanolic extract of *Melaleuca alternifolia* leaves showed that phenolic compounds and flavonoids were found in high concentrations compared with other plant extracts (Table 7). The results of DPPH scavenging activity is relatively compatible with those of total phenolic and flavonoid compounds content, where the highest DPPH activity (95.5%) was determined in *Melaleuca alternifolia* which possessed the highest phenolic and flavonoid compounds content 82.6 and 8.79 mg g^{-1}, respectively.

TABLE 7. Antioxidant activity, phenolic compounds and flavonoids content of methanolic plant extracts in the present survey.

Species	DPPH (%)	Phenolics (mg g^{-1})	Flavonoids (mg g^{-1})
Cassia nodosa	87.1±0.8	65.0±1.0	17.04±4.29
Delonix regia	97.1±0.1	60.3±0.7	4.31±1.33
Kigelia fricana	70.8±7.0	51.0±0.4	6.36±0.75
Ficus sycomorus	95.4±0.0	55.5±0.8	6.11±0.04
Callistemon viminalis	95.4±0.1	70.0±1.8	5.71±0.30
Azadirachta indica	78.8±4.6	62.4±1.3	21.18±0.12
Araucaria heterophylla	95.0±0.3	65.5±0.5	1.99±0.11
Melaleuca alternifolia	95.5±0.0	82.6±3.4	8.79±1.53
Casuarina equisetifola	95.3±0.1	76.3±2.1	6.16±0.87
Bougainvillea glabra	33.2±3.6	51.6±0.3	9.01±0.57
Ficus benjamina	40.7±1.3	59.3±0.0	4.43±0.63
Tamarix aphylla	96.0±0.0	61.3±1.0	4.52±0.68
Magnolia grandiflora	36.2±0.4	65.8±0.3	7.16±0.54
Dalbergia sissoo	64.9±0.3	50.6±1.2	15.78±1.48

The safety of methanolic extract of *Melaleuca alternifolia* in the present survey was partialy determined through FT-IR analysis, as shown in Table 8 and Fig. 4. In the FT-IR spectrum the wave number range of 2000-4000 cm^{-1} showed the absence of toxic cyano (C≡N) group and acetylenic group (C≡C). The selected plant extract was characterized by the presence of aldehyde groups (-CHO) within the wave number range of 2850-2900 cm^{-1} (all were represented with strong peaks). The composition and identification of the fatty acid components present in the methanolic leaf extract of *Melaleuca alternifolia* are shown in Table 9 and Fig. 5. Six compounds were identified: the most abundant compounds were octanoic acid methyl ester (25.67%), 9-Dodecenoic acid, methyl ester, (E) - (24.22%) and n-Hexadecanoic acid (23.98%).

TABLE 8. Functional group Profile of *Melaleuca alternifolia* methanolic leaf extract by FT-IR analysis.

Functional group	Symbol	Range of wave number (cm^{-1})	Presence in *Melaleuca alternifolia*
Hydroxyl group	(OH)	3300-3500	Present
Amino group	(NH_3)	3200-3400	Absent
Aliphatic saturated hydrocarbon chains	(CH_3, CH_2, CH)	2850-2980	Present
Aldehyde group	(CHO)	2850-2900	Present
Carbonyl group	(-C=O)	1700-1750	Present
Aliphatic unsaturated hydrocarbon double bonds	(-C=C-)	1500-1600	Present
Aromatic hydrocarbon ring	--	3100-3200	Absent
Cyano group	(C≡N)	2000-2250	Absent
Acetylenic group	(C≡C)	2000-2250	Absent

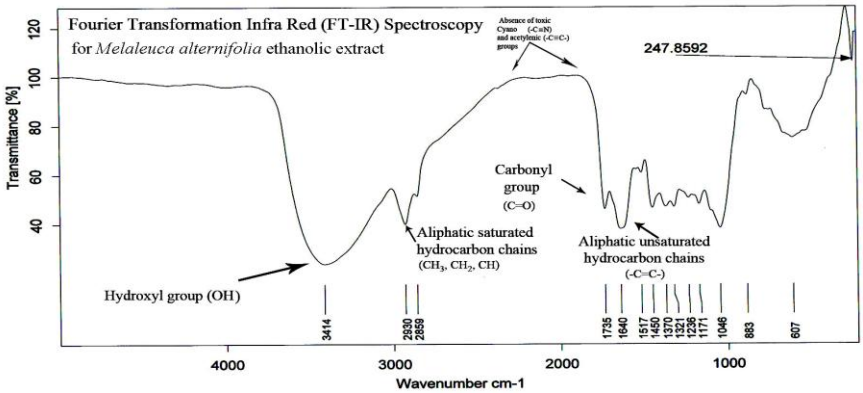


Fig. 4. FT-IR profile analysis for *Melaleuca alternifolia* methanolic leaf extract.

TABLE 9. GC/MS Profile analysis of *Melaleuca alternifolia* methanolic leaf extract.

Pk #	RT (min)	Compound Name	Lipid number	Peak Area	Area (%)
1	10.52	Pentanoic acid, methyl ester	C5:0	214.22	13.93
2	14.86	Heptanoic acid, methyl ester	C7:0	87.143	5.67
3	19.41	Octanoic acid, methyl ester	C8:0	394.64	25.67
4	51.97	Hexadecanoic acid, 15-methyl-, methyl ester	C16:0	100.26	6.52
5	52.60	n-Hexadecanoic acid	C16:0	368.69	23.98
6	54.55	9-Dodecenoic acid, methyl ester, (E)-	C12:0	372.42	24.22
Total				1537.37	100.00

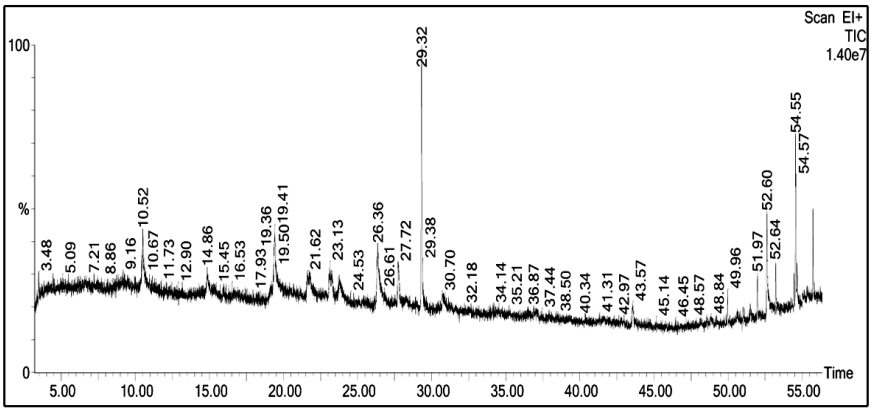


Fig. 5. GC-MS profile analysis for *Melaleuca alternifolia* methanolic leaf extract.

Discussion

Variety of wastes effluent, either with or without partial treatment, discharged into the Nile, results in changes in its physical and chemical conditions and microbial quality. Water pollution is one of the most principal environmental and public health problems in River Nile (El-Amier *et al.*, 2015b). Water temperature is an important factor which affects the biological activities of aquatic organisms (Bojanic *et al.*, 2001) and the amount of oxygen that can be dissolved in water. In the present study, elevation of temperature at the entrance site of Benover village water treatment station could be attributed to the discharge of hot waste water (industrial wastes) into the Nile, which consequently induces thermal pollution leading to elevation of temperature. This finding agrees with Bedair (2006). In addition, the high value of EC in the Nile water recorded in the present study may be attributed to the discharge of great amounts of agricultural effluents containing large amounts of dissolved ions and high amount of organic and inorganic constituents from El-Rahawy and Sobol drains. This was previously confirmed by the study of Elewa and Mahdi (1988). The present results indicated also an apparent increase in the water total dissolved solids, this may be attributed to high content of suspended and dissolved matters. River Nile water pH is always in the alkaline side with slight fluctuations between different stations (Abdo, 2002 and El-Sarnagaway *et al.*, 2010).

The high concentrations of dissolved oxygen (DO) are very vital for aquatic organisms, as it is required for the metabolism of aerobic organisms and organic matter decomposition (Ezzat *et al.*, 2012). Oxygen is also a key component required for all oxidation, nitrification and decomposition processes; and is controlled by three factors: photosynthesis, respiration and exchange at the air-water interface (Krom *et al.*, 1989). Generally, DO concentrations in the Nile are always higher than 7.0 mg L^{-1}, indicating high assimilation capacity (Ezzat *et al.*, 2002). According to APHA (2011), the chemical oxygen demand (COD)

is used as a measure of the oxygen equivalent to organic matter content of a sample that is susceptible to oxidation by a strong chemical oxidant. Generally, during sampling process in winter, the COD value was high at the recorded stations, this may be due to the winter closure which started at the end of December causing decrease in the level of Nile water, leading to elevate the organic load of the Nile during this period.

Reid (1961) reported that ammonia-nitrogen in excess of 1 mg L^{-1} has been given as an indicator of organic pollution, and could be toxic to aquatic species in concentration over 2.5 mg L^{-1}. Higher concentration of ammonia in the area of the present study could be attributed to organic pollution resulted from domestic sewage, industrial waste and fertilizer runoff. The pronounced increase in NH_3 content during winter may be attributed to the decomposed organic matter and dying of algal blooms resulted in increasing of ammonia concentration (Krom *et al,* 1989). Generally, surface water, not influenced by human activities, contains nitrate concentration less than 5 mg L^{-1}. Excess of 5 mg L^{-1} usually indicates pollution by human or animal waste or/and fertilizers run off (Chapman, 1992). At the same time, nitrate is generally the only thermodynamically stable form of nitrogenous compounds in absence of oxygen (Horna, 1972).

Heavy metals have environmental persistence, almost of them have no toxicity at low concentration, but they have ability to incorporate into food chain, and thus it is possible to be concentrated to toxic levels by the aquatic organisms. They are usually divided into two subclasses: Co, Cu, Fe, Mn and Zn which are essential for the correct functioning of biochemical processes; and Hg, Cd, Cr and Pb that have no established biological function and represent the most important contaminants in the aquatic environment. Heavy metal pollution in water is generally associated with agricultural, industrial and municipal discharges (Zaghloul, 2001).

The total bacteria developing at 20-22°C are saprophytic types and non-pathogenic to human beings, while those developing at 37°C are mainly or potentially parasitic types derived from the soil, sewage, or excretal materials (APHA, 2011). In the present study, the total count of bacteria at 37°C was high in winter during the sampling period. However, in Rosetta Branch, Rabeh (2007) reported that the highest bacterial counts (66.8×10^7 and 65.6×10^7 CFU ml^{-1}) at both incubation temperatures (22 and 37°C) were recorded during summer. On the other hand, the highest counts were recorded at Sobol Drain in Menofyia Governorate, while the lowest were at Rosetta Estuary (0.3×10^7 and 0.2×10^7 CFU ml^{-1}). Shaban and El-Taweel (2003) counted *Candida albicans* and total yeast as indicators of fecal pollution in the River Nile. Concerning yeast species contamination to water, Wojcik *et al.* (2003) revealed 28 yeast species representing seven genera: *Candida, Cryptococcus, Geotrichum, Kloeckera, Rhodotorula, Saccharomyces* and *Trichosporon* in the littoral zone of the Sulejów Reservoir of Poland. Kiziewicz (2004) found that the occurrence

of *Candida albicans* and *Candida tropicalis* was associated with high concentration of ammonium nitrogen and suspended substance. These was closely related to species isolated by El-Sarnagaway *et al.* (2010) who made total microbial count including fungi and yeasts as indicators for fecal pollution in the River Nile (Rosetta branch) at Kafr El-Zayat Industrial Area during (2005-2006). The highest count of yeasts ($148x10^2$ CFU ml^{-1}) was recorded during winter at 37°C at about one Km downstream of El-Malh and Soda Company. The isolated yeasts were *Rhodotorula glutinis, Saccharomyces cerevisiae, Candida albicans Candida magnoliae, Candida pelliculosa, Trichosporon mucoides* and *Candida glabrata*. The most common identified fungi were *Trichoderma* spp., *Cladosporium carrionii, Aspergillus tamari, Asperigullus niger* and *Penicillium marneffei.*

Collection of many personal patient data and the recorded medical historic information, indicated that different risk factors were considered to be obvious reasons for the spreading of mycotic ulcers. The present study revealed that the mycotic ulcers were observed to be common among the male patients aged more than 40 years old, that could be explained by increased outdoor activities of males comparing to females in oriental community, which expose males to risks of ocular truma. These findings were relatively close to a previous study in Egypt that recorded a male to female ratio of 9:1 with age range >40 years (AL-Hussaini *et al.*, 1994). Moreover, the study of Moharram *et al.* (1999) revealed that fungal keratitis affected males more than females, and was more frequent in those aged from 20 to 50 years. It is evident that social hygiene parameters had a great effect on the spreading of infectious human mycotic corneal ulcers. Patients holding rural houses possessed higher incidence of mycotic ulcers. On the other hand, those of higher incidence of mycotic ulcers was recorded among patients having indoor pipes water source than outdoor pumps, theses reflect the bad disinfection of the pipes leading to more growth of infectious organisms. Abu El-Souod *et al.* (2013) evaluated the spreading of mycotic infection of human corneal ulcers on the basis of the predisposing factors through studying the epidemiology of human mycotic keratitis in Tanta University, Ophthalmology hospital, they revealed that contact lens users and farmers were found to be more susceptible for mycotic keratitis; also immunocompromised male patients aged more than 40 years with rural residence were the most frequent inhabitants of fungal corneal ulcers.

Martin and Ernst (2004) revealed that traditional medicine had made use of many different plant extracts for treatment of fungal infections. Plants produce variety of medicinal components that can inhibit pathogen growth. A considerable number of studies on medicinal plants and alternative compounds, such as secondary metabolites, phenolic compounds, essential oils and extracts, have been performed (Negri *et al.,* 2014). The medicinal plants received an extensive use in traditional system of medicines all over the world, especially in the rural areas, to cure various health problems. The main reasons for the popularity of herbal medicines are 1- plants are near to nature, hence more

effective than allopathic medicines, 2- they are easily accessible, 3- they are cheaper mode of treatment and 4- show fewer side effects or adverse reactions as compared to modern synthetic drugs (Abbasi *et al.,* 2010). Noumi *et al.* (2011) detected the chemical composition, antioxidant and antimicrobial potential of *Melaleuca alternifolia* because it was used as a traditional treatment of fungal infection, its oil has been used medicinally in Australia, due to its antimicrobial (Mondello *et al.,* 2003) and anti-inflammatory (Hammer *et al.,* 2000).

In the present study, phenolics, flavonoids and fatty acids were found in high concentrations, they act as secondary metabolites of *Melaleuca alternifolia*. The beneficial medicinal and antimicrobial actions of plant resources typically result from the secondary metabolites present in the plant (Jadon and Dixit, 2014). Although, biological activity is usually not attributed to a single compound, but a combination of the metabolites. Wu *et al.* (2006) have shown that phenolic compounds of a plant origin possessed antioxidative and antimicrobial activities. The antioxidant capacity of phenolics is due to their ability to scavenge free radicals, donate hydrogen atoms or electrons or chelate metal cations (Amarowicz *et al.,* 2004). Many previous studies showed highly positive correlations between the total phenolic compounds, the antioxidant capacity of the plant extracts and the antimicrobial activity (Shan *et al.,* 2007). Adamo *et al.* (2004) had pointed out that some environmental conditions are capable of breaking the chemical bonds of polyphenols, thereby releasing soluble phenols of low molecular weights, leading to an increase of antioxidant capacity.

The present study revealed that methanolic extract of *Melaleuca alternifolia* was considered to be effective antifungal agents, its activity has been attributed to the presences of some active functional groups. Sensitivity of *Candida pelliculosa* and *Trichosporon mucoides* to the tested plant extract was in the present study was in accordance with that of Noumi *et al.* (2011).

Many studies have been performed to understand the mechanism of antimicrobial action of fatty acids. It was concluded that fatty acids and their esters exhibited non-specific modes of action (Davidson *et al.,* 2005). The free carboxylic group of the fatty acids is the most important structure required for antimicrobial activity because it allows an optimal insertion into cells that have hydrogen-bond-acceptor groups in the membrane. Hexadecanoic acid was confirmed as antibacterial and antifungal compound (Stojanovi'c *et al.,* 2010). The development of resistance of microbes, including fungi and yeasts, towards antimicrobial agents already in use, necessitates the search for alternative antimicrobials, including fatty acids and their derivatives (*e.g.* methylated and hydroxyl fatty acids). Although fatty acids may not be as effective as chemical fungicides, they possess less environmental risks. They are not only biodegradable, but exhibit a high degree of specificity. In addition, fatty acids are accepted food additives and importantly, pathogenic fungi are less likely to

become resistant to antifungal fatty acids. The most important target of antifungal fatty acids is the cell membrane. They cause an increase in membrane fluidity, which will result in leakage of the intracellular components and cell death (Carolina *et al.,* 2011).

Conclusion

The present study revealed that a high level of chemical and biological contamination was recorded in River Nile at Kafr El-Zayat Industrial Area, Also, it was proved that the infectious diseases, such as mycotic keratities among patients of the surrounding residential area, were closely related to the identified fungal isolates from water samples. A new trial was performed to treat the most common fungal infection (*Candida pelliculosa* and *Trichosporon mucoides*) with a natural product (*Melaleuca alternifolia* methanolic leaf extract) with a promising inhibition rate and effective mode of action.

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تقييم النشاط المضاد للفطريات لبعض المستخلصات النباتية فى مواجهة عدوى الإلتهاب الفطرى لقرنية الإنسان المرتبطة بالمياه

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تتسبب المستويات المرتفعة لملوثات مياه النيل فى تغيير حاد لمؤشرات التلوث مثل درجة الحرارة، تركيز أيون الهيدروجين، الأكسجين الذائب، مستويات الإستهلاك الكيميائى للأكسجين، النشادر، النترات، بالإضافة إلى تركيز بعض العناصر الثقيلة (مثل الرصاص، الزئبق والنحاس)، مما يؤدى إلى عدم صلاحية المياه للشرب والرى ومظاهر الحياة المائية الأخرى. ومن ناحية أخرى تدل المؤشرات الأحيائية الدقيقة على تعرض مياه فرع رشيد للتلوث بالصرف الصحى مع تواجد شائع لعدة أنواع من الخمائر والفطريات (كانديدا بيلليكيولوزا، كانديدا تروبيكاليس، ترايكوسبورون ميوكويديس وأسبيرجيلاس نايجر). وقد ارتبط الإلتهاب الفطرى لقرنية الإنسان والمعدى للمرضى قاطنى المناطق السكنية المحيطة بمنطقة الدراسة، بالخمائر والفطريات المعرفة من عزلات المياه الملوثة. أظهر بوضوح المستخلص الميثانولى لأوراق نبات ميلالوكا ألتيرنيفوليا نشاط مثبط لنمو الخمائر الأكثر شيوعاً فى هذه الدراسة (كانديدا بيلليكيولوزا وترايكوسبورون ميوكويديس)، هذا ما تم تأكيده بالفحص الدقيق للخلايا تحت المجهر الإلكترونى النافذ. كما تم تقدير التركيز الأدنى المثبط وتحديد التركيب الكيميائى للمستخلص النباتى بإستخدام تقنيات الأشعة تحت الحمراء والفصل اللونى الغازى.