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Assessment of the Antifungal Potential of Selected Desert Plant Extracts against Pathogenic Human Fungi

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THE ANTIFUNGAL activities of six selected desert plants belonging to families Chenopodiaceae, Solanaceae, Brassicaceae, Fabiaceae, Zygophyllaceae and Aizoaceae from the North Western Mediterranean coastal region, Egypt, were investigated against six human pathogenic fungal species (*Candida albicans*, *Candida tropicalis*, *Trichosporon* sp., *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus versicolor*). Aqueous as well as organic crude extracts of the selected desert plants were screened against the different human pathogenic fungal species. Results demonstrate that the non-polar fraction of *Atriplex halimus* L. and *Mesembryanthemum crystallinum* L. exhibited the most antagonistic activity. The MIC values of fractions against yeasts and moulds ranged from 0.195 to 6.25mg/ml, whereas the fungicidal activity ranged from 0.781–12.5mg/ml. The most efficient antifungal activity was displayed by the petroleum ether fraction of *M. crystallinum* L. which inhibited the growth of yeast at MIC value of 0.195mg/ml and moulds at MIC values that ranged from 1.56–3.12mg/ml. Notably, the majority of combinations between plant extracts and antifungal drugs and/or plant extracts showed synergistic antifungal activities against the tested fungal species. As for the probable mechanism for the observed antifungal activity of the petroleum ether fraction of *M. crystallinum*, a considerable reduction in the ergosterol content and leakage of plasma and cellular membranes of the tested fungal species.

Keywords: Desert plants, Antifungal activity, Synergistic effect, Plasma membrane, Ergosterol.

Introduction

Despite numerous improvements, infectious diseases still pose a serious challenge to human health. With the ever-increasing numbers of immuno-compromised patients, the prevalence of fungal infections has significantly increased over recent years (Steinbach et al., 2003 and Upton & Marr, 2006). Because of the difficulty of their diagnosis and treatment, fungal infections are a prime cause of morbidity and mortality. They range from wide spread superficial to life-threatening systemic infections (Keller et al., 2015). Among the opportunistic fungal pathogens *Candida albicans* and *Aspergillus fumigatus* are the most common (Steinbach et al., 2003 and Upton & Marr, 2006). For systemic infections, a limited number of antifungal drugs is available, including the commonly known classes such as azoles, polyenes and echinocandins. Yet, several adverse side effects have increasingly been associated with the use of several existing antifungal drugs, including nephrotoxicity, hepatotoxicity and neurotoxicity (Andriole, 1994). In addition to the emerging resistance, the elevated costs for several available antifungals substantiated the need for new ones (Keller et al., 2015).

Over the years, the search for naturally occurring antimicrobials has been the focus of many researches in an effort to replace synthetic drugs (Kelmanson et al., 2000 and Ahmad & Beg, 2001). Traditionally, herbal medicine provides an interesting, largely unexplored source of potential new drugs (Udgirkar et al., 2012 and Gahewal et al., 2014). Because of their ability to produce many secondary metabolites and phytochemicals, plants are generally regarded as a rich source of

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less toxic and more effective antimicrobial agents (Kelmanson et al., 2000 and Ahmad & Beg, 2001). Several studies have demonstrated the effective use of plant extracts for the purpose of screening for novel antimicrobial activities (Guleria & Kumar, 2006; Zakaria et al., 2007 and Kumar et al., 2017).

The aim of the present study was to perform an *in vitro* evaluation of the antifungal potential of extracts from six selected desert plants against selected human fungal pathogens and elucidate the probable mode of action.

Materials and Methods

Collection and identification of plants

The aerial part of the six selected plants (*Atriplex halimus* L., *Alhagi maurorum* Medic, *Brassica tournefortii* Gouan, *Nicotiana glauca* R. C. Grah, *Mesembryanthemum crystallinum* L. and *Pegnaum harmala* L.) were collected in the flowering stage from the North western Mediterranean coastal region, Egypt, during May 2015 and identified by the Herbarium Desert Research Center.

Preparation of crude plant extracts

The ethanolic extracts

Air-dried and finally ground samples (100g) of each plant material were extracted three times with ethanol (70%). The alcoholic extracts were concentrated in vacuum using rotary evaporator at 40°C. (Jacquelina et al., 2005)

The water extracts

The air-dried plant parts were extracted with distilled water. The filtered extracts were then used for screening (Koh, 2009).

Fractionation by different organic solvent

One hundred grams of fine powder of plants were put in the thimble and extracted successively three times with petroleum ether, chloroform, ethyl acetate and methanol for 72h. All the solvent extracts were concentrated using rotary evaporator (BÜCHI VAC V- 500). The extracts were preserved in air tight brown bottle until further use (Nostro et al., 2000).

Microbial isolates

Human pathogenic fungal species; *Candida albicans*, *Candida tropicalis*, *Trichosporon* sp., *Aspergillus fumigatus*, *Aspergillus flavus* and *Aspergillus versicolor* were obtained from Ain Shams Specialized Hospital and identified at the Microbiology Department, Faculty of Science, Ain Shams University.

Inoculum preparation

Yeast inoculum suspensions were prepared as described in the NCCLS M27-A2 method (NCCLS, 2002a). The turbidity was measured with a spectrophotometer at 530nm and was adjusted to match a 0.5McFarland density standard, resulting in a concentration of 1-5x10^6cells/ml. This inoculum was used directly for inoculation of agar plates. For *Aspergillus* spp. inoculum suspensions were prepared from fresh, mature (3 to 5 day-old) cultures grown on Sabouraud agar or potato dextrose agar slants. The colonies were covered with 5ml of sterile distilled water. Tween-20 (5%) was added to facilitate the preparation of the *Aspergillus* inocula. The suspensions were obtained by exhaustive scraping of the surface with a sterile loop. The inoculum size was determined by spectrophotometer reading at a wavelength of 530nm to a transmittance level of 80 to 82%, as described in the NCCLS M38–A (NCCLS, 2002b).

Susceptibility testing

Agar diffusion method

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts (Magaldi et al., 2004). For screening of antifungal activities, the crude ethanol extract and fractions of plants were dissolved in dimethylsulfoxide (DMSO) and then diluted to final concentrations of 200mg/ml and 100mg/ml for crude extract and fractions, respectively, for use in the antifungal assay. The agar plate was surface inoculated by spreading of the microbial inoculum over the entire agar surface using sterile swap. A hole with a 6-8mm diameter was then punched aseptically with a sterile cork borer and a 200µl volume of the antifungal agent or extract solution (at the desired concentration) was introduced into the well, where fluconazole was included as a positive control. The plates were then incubated at 30°C for 24h for *Candida* spp. and 48-72h for *Aspergillus* spp.

Synergy testing

To test for the synergistic effect of a combination of the plant extracts and the tested antifungal drug and a combination of two plant extracts (*Atriplex halimus* recording the highest

inhibition zones with yeast fungi and *Brassica tournefortii*, recording the highest inhibition zone with mould fungi) the agar well diffusion method was adopted (Magaldi et al., 2004).

Determination of the minimum inhibitory concentration (MIC)

The Clinical and Laboratory Standards Institute (CLSI) reference methods for antifungal susceptibility testing against yeasts and filamentous fungi, M27-A3 (CLSI, 2008a) and M38-A2 (CLSI, 2008b), respectively, were applied. The minimum inhibitory concentration (MIC) was determined according to the Clinical Laboratory Standards Institute using 96-wells microtitre plates. 100µl of two-fold diluted fractions and reference drugs in RPMI 1640 (Sigma Aldrich) were added in the wells of the microtitre plate followed by addition of 100µl of yeasts inoculum standardized at 2.5×10^3cells/ml or $0.4–5\times10^4$cells/ml for filamentous fungi. The plates were incubated at 30°C for 24h for *Candida* spp. or 48h for *Aspergillus* spp. The MIC readings were obtained spectrophotometrically with a microplate reader at 492nm (Arikan, 2007). MIC was defined as the lowest concentration that inhibited visible fungal growth.

Determination of the minimum fungicidal concentration (MFC)

The MFC was determined by inoculating 20µl from the wells with concentrations equal to and higher than the MICs on Potato dextrose agar medium and incubate them overnight at 28°C. MFC values were determined as the concentrations that totally inhibited the growth of fungal colonies (Arikan, 2007).

Mode of action studies

Determination of ergosterol content in the plasma membrane

Cellular ergosterol was quantified as described by Tian et al. (2012). The extraction was performed from both yeast and mould strains. Ergosterol was extracted after the exposure of the six different strains to sub-inhibitory concentrations (sub-MIC) of the petroleum ether fraction of *M. crystallinum*. Samples without any treatment were considered as controls. After incubation, fungal cells for each strain were harvested and washed twice with distilled water. The net wet weight of the cell pellet was determined. Five milliliters of 25% alcoholic potassium hydroxide solution was added to each sample and mixed with vortex for 2min followed by incubation at 85°C for 2h. Sterols were then extracted from each sample by adding a mixture of 2ml sterile distilled water and 5ml n-heptane, followed by vigorous mixing by vortex for 2min allowing the layers to separate for 1h at room temperature. The n- heptane layer was analyzed spectrophotometrically. Ergosterol content was calculated as a percentage of the wet weight of the cells and was based on the absorbance and wet weight of the initial pellet. The calculated formula of the ergosterol amount is as follows:

% ergosterol + % 24(28) dehydroergosterol= (A282/290)/pellet weight, % 24(28) dehydroergosterol= (A230/518)/pellet weight and % ergosterol= (% ergosterol + %24(28) dehydroergosterol) - %24(28) dehydroergosterol

where 290 and 518 are the E values (in percentages per cm (determined for *crystallinum* ergosterol and 24(28) dehydroergosterol, respectively) and pellet weight is the net wet weight (g).

Determination of endogenous reactive oxygen species (ROS) production

Endogenous ROS levels of clinical isolates were detected by a fluorometric assay using the fluorescent dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma Chemical Co.) as ROS indicator according to the methods of Kobayashi et al. (2002). The obtained fungal cell suspension of each strain was exposed to the petroleum ether fraction of *M. crystallinum* at sub-MIC value for 12h. Samples without any treatment were considered as controls. After incubation, the fungal cells were centrifuged at 5000×g for 5min and washed twice with phosphate-buffer saline (PBS). The pellet was adjusted to $4x10^6$cell/ml and resuspended in 0.5ml PBS and DCFH-DA (Sigma Chemical Co.) with a final concentration of 10µM and incubated for 2h at 28°C. After incubation, the fungal cells were centrifuged at 5000xg for 5min and washed twice with PBS, the pellet was resuspended in 0.5ml phosphate-buffer saline (PBS). The fluorescence was measured with a fluorometer using excitation and emission wavelengths of 495 and 520nm, respectively.

Effect of the plant fraction on cell membrane integrity

This test was performed to determine the ability of the petroleum ether fraction of *M. crystallinum* to alter the cell membrane integrity of the fungal cell, causing leakage of intracellular molecules,

as described by Cordeiro et al. (2014). The six test strains were exposed to sub-MIC concentrations of the petroleum ether fraction of *M. crystallinum*. Growth control (without plant fraction) and blank solution (culture medium control) were also included in the test. One milliliter from each tube containing the fungal inoculum and exposed to the plant fraction was transferred to sterile microcentrifuge tubes and centrifuged for 15min (13,400×g). Of the supernatant, 70µl were removed from each tube and diluted 1:10 with sterile distilled water and the content was subjected to a spectrophotometric reading at wavelength of 260 to 280nm for analysis of the presence of nucleic acids and proteins, respectively.

In vitro cytotoxicity using the MTT assay

HepaRG cells (obtained from the American Type Culture Collection (ATCC), Manassas, Virginia, USA) were cultured with complete DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (GIBCO, New York, USA) at 37°C for 3 days under 5% CO_2. HepaRG cells in exponential growth phase were washed, trypsinized and suspended in DMEM medium and were seeded at a density of 1×10^4cells/well. The cell density was adjusted by 0.4% trypan blue exclusion method. All compounds were added at different concentrations (20, 40, 80, 160, 320mg/ml) to the wells and the cells were cultured for 24h in a 5% CO_2 with 95% humidity incubator. The medium was washed gently twice with ice-cold PBS and a volume of 200µl MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole (Molecular probes, Eugene, Oregon, USA; Cat.no.V-13154)) was added to each well. The microplate was incubated at 37°C for another 4h in CO_2 incubator. About 180µl medium/MTT were removed and 100µl acidified isopropanol were added per well to solubilize the formazan produced. Finally, the microplate was incubated with shaking for 15min and a microplate reader (Stat Fax-2100, Awareness Technology, Inc., USA) was used to measure the absorbance of each well at 570nm. Assays were performed in triplicate on three independent experiments. Sigmoidal and dose dependent curves were constructed to plot the results of the experiment. The concentration of the compounds inhibiting 50% of cells (IC50) was calculated using this sigmoidal curve.

Statistical analysis

Data were analyzed using statistical program for social science (SPSS) version 20.0. (Leslie, 1991). the following tests were done:

Independent-samples t- test of significance was used when comparing between two means. A one way analysis of variance (ANOVA) when comparing between more than two means.

The confidence interval was set to 95% and the margin of error accepted was set to 5%. So, the probability (P value) was considered significant as the following:

P value≤ 0.05 was considered as significant.

P value≤0.001 was considered as highly significant.

P value>0.05 was considered as insignificant.

Results

Susceptibility testing

The antifungal properties of the examined ethanolic extracts of the selected desert plants against several human pathogenic fungal species are presented in Table 1. The obtained results indicated that crude extracts of both *M. crystallinum* and *Peganum harmala* have the most potent antifungal activities against all tested pathogenic fungi followed by *Nicotiana glauca* and *Atriplex halimu*s which demonstrated antifungal activity against four only of the tested fungal species, *Alhagi maurorum* which demonstrated antifungal activity against three of the tested pathogenic fungi and finally *Brassica tournefortii*, which inhibited only two fungal species. No inhibition was recorded using water extracts. It was noted that there is a statistical correlation between desert plant species and inhibition zone diameter.

The antifungal properties of the various organic (petroleum ether, chloroform, ethyl acetate and methanol) extracts of each plant were then examined, at a sample concentration of 100mg/ml, using the agar well diffusion method (Table 2). The results indicate that petroleum ether fractions of *Mesembryanthemum crystallinum*, *Nicotiana glauca*, *Peganum harmala*, *Atriplex halimus* and *Brassica tournefortii* are the most active fractions compared with chloroform, ethyl acetate and methanol fractions and also with crude ethanolic extracts. Among the several examined petroleum ether fractions, *Mesembryanthemum crystallinum* demonstrated the highest antifungal activity. *Trichosporon* was the most sensitive species followed by *Candida albicans, Candida tropicalis,*

Aspergillus flavus, Aspergillus fumigatus and finally *Aspergillus versicolor*.

It is worth mentioning that although *Brassica tournefrtii* showed weak antifungal activity, it recorded the highest inhibition zone against *A. fumigatus* (40mm) and *A. flavus* (45mm) using petroleum ether. Results showed statistically significant difference between solvent in each of the plant fractions and inhibition zone diameters (representing antifungal activity).

Synergistic evaluation of the different plant fractions with fluconazole

The synergistic effect of most active plant fractions and fluconazole was tested using well diffusion assay (Table 3). Results indicate that the majority of combinations between plant extracts and fluconazole showed highly synergistic effect against all tested fungal species except the combination between *Alhagi maurorum* extract and fluconazole which showed strong antagonistic effect against *A. fumigatus* and *A. versicolor*.

The Synergistic antifungal activity of petroleum ether fraction of *Brassica tournefortii* and *Atriplex halimus* was also evaluated (Table 4). The synergistic effect of the combination between the two plant extracts was variable. There was a noticeable synergistic effect observed against *C. albicans* and *C. tropicalis*, a slight synergistic effect with *Tricosporon* and *A. versicolor* and antagonistic effect with *A. flavus.*

Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

According to the MIC value, there was a variable effect between yeast and filamentous fungi (Table 5). *C. albicans*, *C. tropicalis* and *Trichosporon* sp. were sensitive to the petroleum ether extract of *M. crystallinum* with MIC value of 0.195mg/ml. On the other hand, *A. fumigatus* was sensitive with the least MIC value of 1.56mg/ml while *A. flavus* and *A. versicolor* were resistant with MIC value of 3.125mg/ml. In addition to the result of *M. crystallinum*, *C. tropicalis* was sensitive to both fractions of *A. halimus* and *A. maurorum* with MIC value of 0.195mg/ml. Generally, results of Table 5 showed a pattern of sensitivity of the yeast strains and resistance of the filamentous strains.

Concerning the MFC test, *C. tropicalis* was the resistant yeast strain against *B. tournefortii* with MFC value of 6.25mg/ml while the MFC value of the other yeast strains were less and ranged from 0.39 to 3.125mg/ml. Filamentous strains showed moderate resistance of MFC value of 6.25mg/ml and high resistance MFC value of 12.5mg/ml, while *A. fumigatus* was the only strain with low resistance with MFC of 3.125mg/ml against *M. crystallinum* (Table 6).

TABLE 1. Inhibition zone diameter of the ethanolic crude extracts of desert plants against human pathogenic fungal species using agar well diffusion method.

Tested fungal species / Desert plant species	*C. albicans*	*C. tropicalis*	*Trichosporon* sp.	*A. fumigatus*	*A. flavus*	*A. versicolor*	Average zone of inhibition (mm)
	Inhibition zone diameter (mm)						
Mesembryanthemum crystallinum	22	12	20	18	16	18	18
Nicotiana glauca	15	16	25	12	0	0	11
Peganum harmala	23	13	26	25	20	25	22
Atriplex halimus	10	21	21	11	0	0	11
Alhagi maurorum	0	16	24	0	13	0	9
Brassica tournefortii	0	22	22	0	0	0	7
Positive control (Fluconazole) (µg/ml)	27	23	16	25	20	29	23
Negative control (DMSO)	0	0	0	0	0	0	0
Water extract	0	0	0	0	0	0	0
ANOVA	9.546	13.146	23.529	44.708	19.043	9.096	14.901
P value	**0.049**	**0.022**	**0.003**	**<0.001**	**<0.001**	**0.028**	**0.011**

C, *Candida*; *A*, *Aspergillus*.

TABLE 2. Inhibition zone diameters of desert plants extract fractions against human pathogenic fungal species using agar well diffusion method (mm).

Tested fungal species / Plant extract	*C. albicans*	*C. tropicalis*	*Trichsporn* sp.	*A. fumigatus*	*A. flavus*	*A. versicolor*
	Inhibition zone diameter (mm)					
Mesembryanthemum crystallinum						
Petroleum ether extract	35	32	30	40	35	19
Chloroform extract	15	23	27	0	40	0
Ethyl acetate extract	30	13	20	0	0	0
Methanol extract	20	0	20	0	0	0
ANOVA test	10.00	7.971	2.866	37.089	26.875	6.760
P value	**0.019**	**0.029**	**0.239**	**<0.001**	**<0.001**	**0.009**
Nicotiana glauca						
Petroleum ether extract	27	24	35	22	32	18
Chloroform extract	18	19	28	0	25	0
Ethyl acetate extract	0	22	30	15	0	22
Methanol extract	0	0	23	0	0	0
ANOVA test	14.680	9.667	2.552	10.429	22.900	12.182
P value	**0.002**	**0.022**	**0.466**	**0.005**	**<0.001**	**0.002**
Peganum harmala						
Petroleum ether extract	35	26	30	22	22	15
Chloroform extract	30	17	25	0	0	0
Ethyl acetate extract	24	16	35	0	0	15
Methanol extract	0	0	22	0	0	0
ANOVA test	22.000	13.785	3.500	10.704	10.704	17.857
P value	**<0.001**	**0.003**	**0.321**	**0.004**	**0.002**	**<0.001**
Atriplex halimus						
Petroleum ether extract	35	28	30	40	20	32
Chloroform extract	22	20	28	0	0	15
Ethyl acetate extract	24	12	30	0	0	20
Methanol extract	15	0	11	0	0	0
ANOVA test	8.385	18.262	37.515	23.170	9.003	21.004
P value	**0.035**	**<0.001**	**<0.001**	**<0.001**	**0.003**	**<0.001**
Alhagi maurorum						
Petroleum ether extract	0	0	23	0	0	0
Chloroform extract	0	0	21	0	0	0
Ethyl acetate extract	0	10	30	0	12	0
Methanol extract	0	0	20	0	0	0
ANOVA test	0.000	1.667	2.596	0.000	7.143	0.000
P value	**1.000**	**0.197**	**0.458**	**1.000**	**0.008**	**1.000**
Brassica tournefortii						
Petroleum ether extract	0	22	30	40	45	28
Chloroform extract	0	19	22	0	0	0
Ethyl acetate extract	0	16	15	0	0	22
Methanol extract	0	14	0	0	0	0
ANOVA test	0.000	2.070	18.778	27.222	32.001	15.527
P value	**1.000**	**0.558**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

C, Candida; A, Aspergillus.

TABLE 3. Synergistic effect of different plant extracts and fluconazole.

Tested fungal species / Combination		*C. albicans*	*C. tropicalis*	*Trichosporon* sp.	*A. fumigatus*	*A. flavus*	*A. versicolor*
		Inhibition zone diameter (mm)					
Mesembryanthemum crystallinum	Petroleum ether						1
Plant extract		35	32	30	40	35	9
Fluconazole		27	23	16	25	20	29
Mix: Plant extract +Fluconazole		38	32	39	43	36	35
ANOVA test		1.940	19.322	9.482	5.167	5.297	4.723
P value		**0.379**	**<0.001**	**0.009**	**0.076**	**0.071**	**0.094**
Nicotiana glauca	Petroleum ether						
Plant extract		27	24	35	22	32	18
Fluconazole		27	23	16	25	20	29
Mix: Plant extract +Fluconazole		30	35	38	25	33	26
ANOVA test		6.857	3.244	9.596	10.889	3.694	2.658
P value		**0.009**	**0.198**	**0.008**	**<0.001**	**0.158**	**0.285**
Peganum harmala	Petroleum ether						
Plant extract		35	26	30	22	22	15
Fluconazole		27	23	16	25	20	29
Mix: Plant extract +Fluconazole		37	35	38	27	30	15
ANOVA test		1.697	2.786	8.857	0.514	2.333	0.017
P value		**0.428**	**0.248**	**0.012**	**0.774**	**0.311**	**0.897**
Atriplex halimus	Petroleum ether						
Plant extract		35	28	30	40	20	32
Fluconazole		27	23	16	25	20	29
Mix: Plant extract +Fluconazole		37	35	30	42	24	31
ANOVA test		1.697	2.535	25.474	4.841	4.002	0.152
P value		**0.428**	**0.282**	**<0.001**	**0.089**	**0.046**	**0.927**
Alhagi maurorum	Ethyl acetate						
Plant extract		0	10	30	0	12	0
Fluconazole		27	23	16	25	20	29
Mix: Plant extract +Fluconazole		28	16	35	0	15	0
ANOVA test		25.107	5.184	7.185	19.593	2.085	23.516
P value		**<0.001**	**0.075**	**0.028**	**<0.001**	**0.353**	**<0.001**
Brassica tournefortii	Petroleum ether						
Plant extract		0	22	30	40	45	28
Fluconazole		27	23	16	25	20	29
Mix: plant extract +Fluconazole		30	35	35	42	45	30
ANOVA test		26.310	3.925	7.185	4.841	44.545	0.069
P value		**<0.001**	**0.141**	**0.028**	**0.089**	**<0.001**	**0.966**

C, Candida; A, Aspergillus.

TABLE 4. Synergistic antifungal activity of petroleum ether extracts of *Brassica tournefortii* and *Atriplex halimus*.

Tested fungal species / Plant species	*C. albicans*	*C. tropicalis*	*Trichsporon* sp.	*A. fumigatus*	*A. flavus*	*A. versicolor*
	Inhibition zone diameter (mm)					
Atriplex halimus	35	28	30	40	20	32
Brassica tournefortii	0	22	30	40	45	28
Mix oil	38	32	31	40	36	35
ANOVA test	34.243	1.854	9.242	0.000	9.525	0.779
P value	**<0.001**	**0.396**	**0.002**	**1.000**	**0.009**	**0.677**

C, Candida; A, Aspergillus.

TABLE 5. MIC of the most active desert plants extracts on tested human fungal species.

Tested fungal species / Plant fractions	*C. albicans*	*C. tropicalis*	*Trichsporn* sp.	*A. fumigatus*	*A. flavus*	*A. veriscolor*
	MIC value (mg/ml)					
Mesembryanthemum crystallinum (pet. ether)	0.195	0.195	0.195	1.56	3.125	3.125
Nicotiana glauca (pet. ether)	0.781	0.39	0.781	6.25	6.25	3.125
Peganum harmala (pet. ether)	1.56	0.781	0.781	6.25	6.25	3.125
Atriplex halimus (pet. ether)	0.39	0.195	0.39	3.125	3.125	6.25
Alhagi maurorum (ethyl acetate)	0	0.195	0.781	0	3.125	0
Brassica tournefortii (pet. ether)	0	0.39	0.781	6.25	6.25	6.25
Standard drug (Fluconazole)	0.0094	0.0047	0.037	0.037	0.037	0.037
ANOVA test	5.111	14.690	13.364	20.957	5.473	16.478
P value	**0.276**	**0.002**	**0.004**	**<0.001**	**0.033**	**<0.001**

C, Candida; A, Aspergillus.

TABLE 6. MFC of the most active desert plants fractions on tested human fungal species.

Tested fungal species / Plant fractions	*C. albicans*	*C. tropicalis*	*Trichsporn* sp.	*A. fumigatus*	*A. flavus*	*A. veriscolor*
	MFC value (mg/ml)					
Mesembryanthemum crystallinum (pet. ether)	0.781	0.781	1.56	6.25	3.125	6.25
Nicotiana glauca (pet. ether)	3.125	3.125	1.56	12.5	6.25	6.25
Peganum harmala (pet. ether)	3.125	3.125	1.56	12.5	12.5	12.6
*Atriplex halimus (*pet. ether)	3.125	1.56	1.56	12.5	3.12	6.25
*Alhagi maurorum (*ethyl acetate)	0	3.125	3.12	0	12.5	0
Brassica tournefortii (pet. ether)	0	6.25	1.56	12.5	12.5	12.5
Standard drug (Fluconazole)	0.037	0.075	0.075	0.15	0.15	0.15
ANOVA test	27.100	10.103	5.769	80.372	72.592	13.933
P value	**<0.001**	**0.039**	**0.042**	**<0.001**	**<0.001**	**0.003**

C, Candida; A, Aspergillus.

Effect of the petroleum ether fraction of M. crystallinum on plasma membrane ergosterol content

Ergosterol is an essential functional component of the plasma membrane. The effect of sub-inhibitory concentration of petroleum ether fraction of *M. crystallinum* on this vital content is shown in Table 7. There was a decrease in ergosterol content of the tested strains. Concerning yeast, the greatest decrease in ergosterol percentage was recorded with *C. albicans* (67.3%), while for filamentous fungal strains the greatest decrease was recorded with *A. versicolor* (68.3%).

Effect of the petroleum ether fraction of M. crystallinum on endogenous ROS production

Figure 1 shows that treatment of test strains with sub-inhibitory concentration of the petroleum ether fraction of *M. crystallinum* leads to a noticeable increase in ROS in the yeast strain compared with the control. The increase corresponded to 2.7, 1.9 and 2.8 times with *C. albicans*, *C. tropicalis* and *Trichosporon*, respectively. On the other hand, the increase was remarkable in the filamentous strains, recording 6.6, 18 and 6.2 times with *A. fumigatus*, *A. flavus* and *A. versicolor*, respectively.

TABLE 7. Percent reduction of ergosterol content in tested fungal species by petroleum ether fraction of *M. crystallinum.*

Tested fungal species	Amount of ergosterol (%)		Percentage of reduction	t-test	P value
	Control	After treatment			
C. albicans	1.1	0.36	67.3	4.680	<0.001 (HS)
C. tropicalis	1.8	1.02	43.3	4.933	<0.001 (HS)
Trichsporn sp.	1.3	0.57	56.2	4.417	<0.001 (HS)
A. fumigatus	0.9	0.31	65.6	3.731	0.006 (S)
A. flavus	0.8	0.45	43.8	2.214	0.033 (S)
A. versicolor	0.6	0.19	68.3	2.593	0.013 (S)

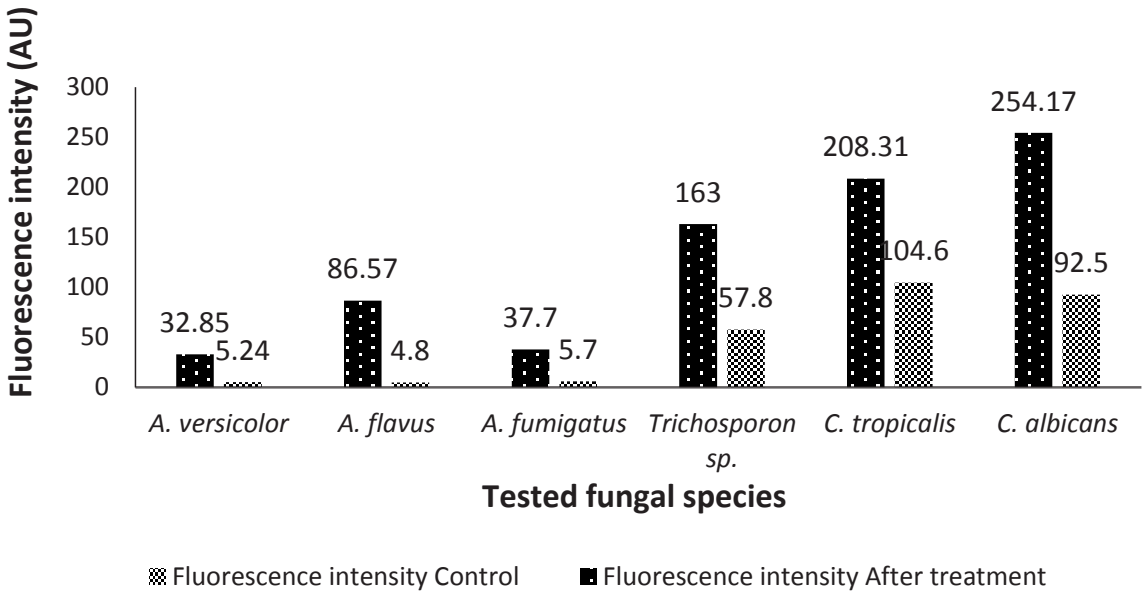

Fig. 1. Effect of petroleum ether fraction of *M. crystalline* on the generation of endogenous ROS.

Effect of sub-MIC of petroleum ether fraction of M. crystallinum on cell membrane integrity

The results indicated that the petroleum ether fraction of *M. crystallinum* was able to induce leakage of the intracellular molecules in the tested fungal strains. The petroleum ether fraction induced the leakage of nucleic acids, which caused a significant increase in absorbance readings at 260nm, compared to readings obtained from the fungal growth control at MIC/2 (Fig. 2). In addition, the petroleum ether fraction also induced leakage of proteins, which caused a significant increase in absorbance readings at 280nm, compared to readings obtained from the fungal growth control (Fig. 3).

Finally, the cytotoxic activities of petroleum ether fraction of *M. crystallinum* were analyzed on the human hepatocyte cell line HepaRG cells and showed IC50 at a concentration of 155900*μ*g/ml (Fig. 4).

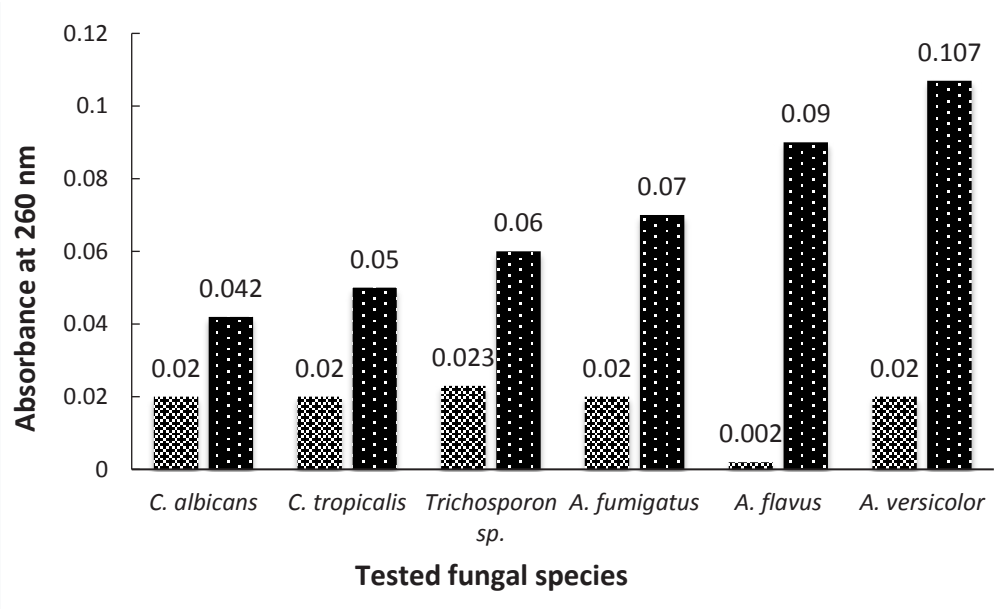

Fig. 2. Effect of petroleum ether fraction of *M. crystallinum* on nucleic acid content.

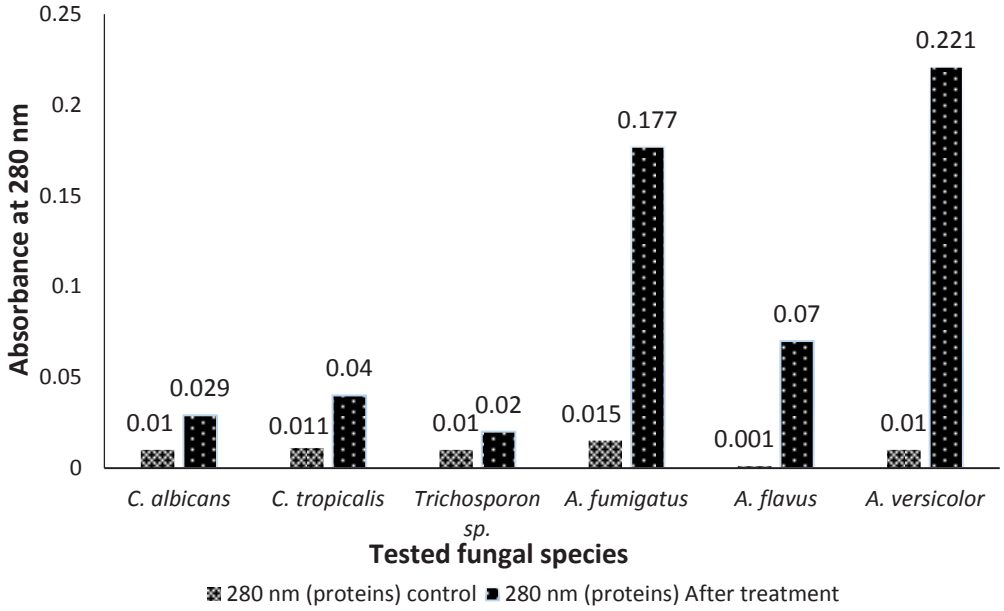

Fig. 3. Effect of petroleum ether fraction of *M. crystallinum* on protein content.

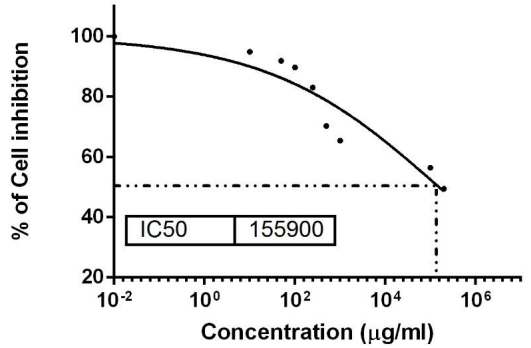

Fig. 4. The cytotoxicity of the petroleum ether fraction of *M. crystallinum.*

Discussion

Incidence of microbial infections has increased in recent decades, especially mycoses which account for a high rate of death among patients with immunosuppressed system. However, only small numbers of effective antifungal drugs are available, including amphotericin B. Yet, the emergence of side effects coupled with the high cost may complicate the treatment of these infections. Hence, there is a pressing need to uncover new antifungal drugs or compounds (Royce & Hans, 2007; González, 2009 and Wheat et al., 2009).

In an attempt to search for more effective and much safer antifungal compounds and because of the well-known therapeutic potential of plant-based compounds, extracts from six selected desert plants belonging to six different families from the Western Mediterranean coastal region of Egypt were tested for their antifungal properties against six human pathogenic fungal species.

Contrary to the well-known perception that traditional healers use primarily water, no inhibition was observed using water extracts from any of the tested plants, which may be attributed to low concentration of antifungal compound in water extracts. This is in agreement with Tuney et al. (2006) and Ponnanikajamideen et al. (2014) who found that organic solvents always provide higher efficiencies in extracting compounds for antimicrobial activity.

Varying degrees of inhibition zones for the extracts and fractions were observed in the present results. This is mainly because the solubility of secondary metabolites is highly dependent on the polarity of the solvents (Das et al., 2010). The inhibitory potentials of ethanolic extracts might be due to the superior solubility of active antimicrobial constituents in ethanol. This finding is consistent with that of Ayyasamy et al. (2012) who reported that better inhibitory potential was obtained using ethanolic extract of *Pleurotus florida*.

The findings of the present study indicate that ethyl acetate extract of *Alhagi* exhibited the highest antifungal activity against the tested fungi as compared to the low polar solvents (chloroform and petroleum ether) which have weak activity on the tested fungi. This agrees with Eloff (1998) and Mohamed & El-Hadidy (2008) who reported that compounds extracted with an intermediate polarity solvent (ethyl acetate, EtOAc) had the highest inhibitory effect as a result of the solvent's ability to extract and isolate active plant metabolites with documented antifungal activity, such as alkaloids, phenolic, flavonoids and terpenoids. On the contrary, petroleum ether fraction was the most active fraction against tested fungal species and the petroleum ether fraction of *M. crystallinum* was the most potent extracts compared to other plants. The presence of different compounds with several biological activities enhances the possibility to use *M. crystallinum* plant as a source for antifungal agent(s) in pharmaceutical and medicinal applications. Similarly, a previous report indicated that the peteroleum ether fraction of *Warburgia ugandensis* was found to be of superior antimicrobial activity, and accordingly the potential of *Warburgia ugandensis* in modern drug formulations was considered (Merawie et al., 2013). This result might indicate the non-polar nature of the active compound which is found in the aerial part of the plants. This may be attributed to the strong ability of high polar solvents to extract higher concentrations of bioactive molecules such as phenolics and terpenoids (Caunii et al., 2012).

Previous reports have indicated the potential benefits of the combined antimicrobial therapy for the treatment of mixed infections (Levinson & Jawetz, 2002). Our results showed that the combination of the plant fraction *M. crystalinum* and antifungal fluconazole demonstrated the highest synergistic effect among the other combinations against all tested organisms. Such synergistic effects with other plant extracts and antifungal or antibacterial agents were reported by other researchers (Kinghorn, 2001; Vattem et al., 2005; Kamatou et al., 2006 and Akinnibosun & Edionwe, 2015). Other combinations in our

results showed a slight decrease or equal to the activity of the plant fraction, this was consistent with Vyas et al. (2015) who found that effect of the combination between the antifungal drug Amphtoericin B and the methanol extract of fifteen medicinal Plants was synergistic against *C. albicans* but was antagonistic against *A. niger*.

As for the effective inhibitory concentration, petroleum ether extract of *M. crystallinum* successfully inhibited the growth of the fungi at a concentration that was comparable to that of fluconazole control, with *Candida* spp., the most common opportunistic pathogens in human systems, being the most sensitive. This result is similar to that reported by Omoruyi et al. (2014) who found that the antifungal activity of the *Mesembryanthemum edule* extract was analogous to that of nystatin and amphotericin B used as controls.

The abovementioned results support the use of these plants in the treatment of microbial diseases. These results are in agreement with those obtained by Abd-Ellatif et al. (2011), who reported the use of plant extracts of *Mesembryanthemum crystallinum*, *Atriplex halimus*, *Carduus getulus*, *Nicotiana glauca* and *Alhagi maurorum*, in the treatment of fungal infections. It also demonstrates their antifungal potential that could be very valuable in our search for novel herbal antimycotic agents.

In the present study, quantitative determination of the ergosterol (an important component of the fungal cell membrane that is crucial for the fluidity and permeability (Espenshade & Hughes, 2007) content of the tested fungal species was adopted as an indicative of the effect of the petroleum ether fraction of *M. crystallinum* on the ergosterol and 24(28) dehydroergosterol of the tested fungal species. Our results revealed that the petroleum ether fraction induced a significant reduction in the ergosterol content of the test organisms. This result suggests that the plasma membrane is considered as an ideal target of *M. crytallinum* extract.

Because of their ability to induce growth inhibition and death of mammalian cells (Benhar et al., 2001), detection of ROS contents (Bonini et al., 2006) was regarded as an important marker of cell viability (Kobayashi et al., 2002 and Pozniakovsky et al., 2005). In the present study, a significant increase in ROS was evident following treatment with the petroleum ether fraction. Similar observations were reported previously, implicating the induction of ROS formation in the antifungal action of many antifungal agents, especially against Candida species (Kobayashi et al., 2002; Cheng et al., 2003; Leiter et al., 2005; Wu et al., 2009; Yan et al., 2009; Qi et al., 2010 and Mello et al., 2011).

Our results suggest that the *Mesembryanthum* active fraction targets the cell and nuclear membranes, as evident by the leakage of proteins and nucleic acids. These results are in agreement with those of Brilhante et al. (2016) who demonstrated that tyrosol caused the leakage of protein and nucleic acids through its effect on both cellular and nuclear membrane. Their results also demonstrated the ability of tyrosol to inhibit the synthesis of ergosterol. Notably, according to our results, the MIC values found of petroleum ether were much lower than the IC50 indicating that petroleum ether fraction of *M. crystallinum* displayed the highest activity against six strains of clinical isolates tested with the lowest cytotoxicity effect.

Conclusion

The present investigation demonstrated that various solvent extracts of the aerial part of six selected plants, *Atriplex halimus*, *Nicotiana glauca*, *Brassica tournefortii*, *Alhagi maurorum*, *Pegnaum harmala* and *M. crystallinum* have variable antifungal properties against several pathogenic fungal species. This could be attributed to the difference in the type and concentration of the various metabolites present in each extract as well as the extraction capacity of the solvents. Importantly, some phytochemicals exhibited their antimicrobial action only when combined with other phytochemical constituents in a synergistic way. This confirms the importance of the synergistic use of medicinal plant extracts to resolve the dilemma of drug resistance and to treat the emerging diseases caused by bacterial and fungal pathogenic species. Finally, since these plants are common in Egypt, these species may have a great potential for use as herbal chemotherapeutics for mycosis.

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تقييم القدرة المضادة للفطريات لمستخلصات نباتية صحراوية مختارة ضد فطريات بشرية ممرضة

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تم دراسة الأنشطة المضادة للفطريات لستة نباتات صحراوية مختارة تنتمي إلى عائلات Chenopodiaceae Zygophyllaceae ، Fabiaceae ،Brassicaceae ،Solanaceae و Aizoaceae من المنطقة الساحلية لشمال غرب البحر الأبيض المتوسط ، مصر، ضد ستة أنواع فطرية ممرضة للإنسان *Candida albicans* و *Candida tropicalis* ،*Trichosporon* sp. ،*Aspergillus fumigatus* ،*Aspergillus flavus* *Aspergillus versicolor*. تم فحص المستخلصات المائية والعضوية من النباتات الصحراوية المختارة ضد أنواع مختلفة من الفطريات البشرية الممرضة. وقد أوضحت النتائج أن الجزء غير القطبي من *Atriplex halimus* L. و *Mesembryanthemum crystallinum* L. يظهر أكثر نشاط مضاد للفطريات. حيث تراوحت قيم أقل تركيز مثبط من المستخلصات ضد الخمائر والفطريات الخيطية من 0.195 إلى 6.25ملغم/مل، في حين تراوحت قيم النشاط القاتل للفطريات من 0.781 - 12.5ملغم/مل. وقد أظهر مستخلص أثير البترول لنبات *M. crystallinum* L. أكثر نشاط مضاد للفطريات حيث أدى إلى تثبيط نمو الخميرة بأقل تركيز مثبط بقيمة 0.195ملغم/مل والفطريات الخيطية بقيمة تراوحت بين 1.56 و 3.12ملغم/مل. ومن الجدير بالذكر أن غالبية التوليفات بين المستخلصات النباتية والعقاقير المضادة للفطريات و/أو المستخلصات النباتية قد أظهرت أنشطة تآزرية مضادة للفطريات ضد الأنواع الفطرية المختبرة. أما بالنسبة للآلية المحتملة للنشاط الملحوظ المضاد للفطريات لمستخلص أثير البترول لنبات *M. crystallinum* L.، فقد حدث انخفاض كبير في محتوى الإرجوستيرول وتسرب البلازما والأغشية الخلوية للأنواع الفطرية المختبرة.