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New Remedy to Control Human Skin Fungal Infections by Silver Nanoparticles Biosynthesized by Two Marine Macro Algae

Anwer El-Badry[(1)], Susan Assawah[(1)], Hala El-Kassas[(2)], Doaa Hegab[(3)], Dalia Amer[(1)]

[(1)]Botany Department, Faculty of Science, Tanta University, Tanta, Egypt; [(2)]National Institute of Oceanography and Fisheries, Alexandria, Egypt; [(3)]Dermatology Department, Faculty of Medicine, Tanta University, Tanta, Egypt.

FROM Dermatology Department of Tanta University Hospitals during 2016, it was observed that cutaneous fungal infections were more common in patients dealing with indoor closed pipe networks as a water source (recorded in 73 cases), sewage disposal by outdoor conservancy (68 cases), of 2-20 years old (38 cases) and house wives (23 cases). The most common fungal isolates among the studied cutaneous infections were *Microsporum canis* (21 cases), *Malassezia furfur* (16 cases), *Trichophyton rubrum* (11 cases), *Candida albicans* (9 cases), *Epidermophyton floccosum* (8 cases) and *Candida tropicalis* (7 cases); that caused Tinea capitis (29 cases), Tinea unguium (23 cases), Tinea versicolor (16 cases) and Tinea corporis (13 cases). The present study reported an eco-friendly biosynthesis of stable silver nano-particles (AgNPs) by two selected marine algal extracts; which gave a great antifungal activity against the most commonly isolated fungi; that was confirmed by their low MIC values, namely MIC for AgNPs of *Corallina mediterranea* was 0.25mg/ml against *Epidermophyton floccosum*, while MIC for AgNPs of *C. officinalis* was 0.5mg/ml against *Candida tropicalis* and further elucidated by their destructive effects, as observable thinning of cell wall (as low as 89.1nm in *C. tropicalis* and 54.9nm in *E. floccosum*), agglutination of cellular proteins, rupture of cell membrane and leakage of intracellular components, revealed through TEM examinations.

Keywords: Cutaneous mycosis, *Candida tropicalis*, *Epidermophyton floccosum*, *Corallina officinalis*, *C. mediterranea*, TEM, AgNPs.

Introduction

Mycoses are classified as human fungal infections caused by eumycotic organisms such as yeast-like fungi, dimorphic fungi, dermatophytes spp., and some other filamentous species (Hay et al., 2010). They occur at different levels which may be cutaneous, subcutaneous, systemic or ocular mycoses; cutaneous infections are superficial fungal infections of the skin, hair and nails that are the most common fungal diseases in humans and affect about 25% (or about 1.7 billion) of the general population worldwide and mainly caused by dermatophytic members of the genera *Trichophyton, Microsporum* and *Epidermophyton*. (Seebacher et al., 2008).

Cutaneous fungal infections may cause different symptoms, such as Tinea capitis (scalp infection) which is recorded as a common dermatophyte infection primarily affecting children, transmitted indirectly via fallen hair and desquamated epithelial cells more often than by direct contact by contaminated barbershop instruments, hairbrushes, combs and shared hats (Ghannoum et al., 2000). It has a worldwide distribution with a high rate (20-50%) in tropical and subtropical regions (Tarazooie et al., 2004). Tinea corporis (neck, arm and leg infection) occurred in wrestlers which infection spreads via skin to skin and wrestling mats (Aghamarian & Ghiasian, 2011). Tinea unguium (Onychomycosis; nail infection of the hand or foot), possesses a prevalence of at least 12.4% in Europe (Hay et al., 2010). Also, Tinea versicolor is a mild and chronic superficial mycotic infection, involved under some exogenous and endogenous predisposing factors, in which fungus can convert from yeast to a pathogenic mycelial form (He et al., 2008). Several factors, such as age, gender, climate, local

[#]Corresponding author emails: ase_science@yahoo.com, anwer.elbadry@science.tanta.edu.eg Tel: 01222806275

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environmental factors, malnutrition and genetic factors influence course of disease. Several reports showed that hot, humid conditions and hygiene are responsible factors for presenting cutaneous infections (Pappas et al., 2009).

Traditional treatments of fungal infection possess some disadvantages, as terbinafine may cause gastrointestinal disturbance (e.g. nausea, dyspepsia and diarrhea), allergic skin reactions (e.g. urticaria), disguisia (unpleasant sense of taste), headache, joint and muscle pain and not recommended for people with liver disease (Mohrenschlager, 2005). Pathogenic *C. albicans* strains may resist azole derivatives, acting as an important clinical problem; also, itraconazole is contraindicated in patients with heart failure or ventricular dysfunction; in the meantime, deep and opportunistic mycoses are considered as an important reason of leukemia, solid cancers and other blood systemic diseases (Tyczkowska et al., 2014).

In the 21st century, nanotechnology is the newly emerging multidisciplinary research area with synthesis of nano-sized materials, playing a top most role in the field of nanomedicines such as health care, medical diagnostic purposes, drug delivery systems, gene therapy applications, and tissue engineering configuration (Amin et al., 2012). Nanoparticles have a greater surface area per weight than larger particles to be more reactive with certain molecules that make them useful or being evaluated for use in many fields (Kathiresan et al., 2009). Although the nanoparticle (NPs) have synthesized by chemical and electrical methods, the biological methods are eco-friendly and have many advantages. Green synthesis of silver nanoparticles (AgNPs) by algae and algal extracts is more advantageous because it is more suitable for large scale production of AgNPs (Singh et al., 2012). Due to its abundance and ready availability, marine algae are good and cost-effective renewable resources of natural phytochemicals that can be exploited for the synthesis of different metallic nanoparticles (Singaravelu et al., 2007).

The present survey aims to evaluate the incidence of cutaneous fungal infections, their predisposing factors and their causative agents among patients of the residential regions related to Tanta University Hospitals. Also, the antifungal activity of biologically synthesized silver nanoparticles by naturally available macroalgae from Egyptian north coast will be evaluated against the isolated fungi.

Materials and Methods

Survey of predisposing factors and the incidence of human cutaneous fungal infections during 2016

A survey was carried out during the period from January 2016 to December 2016. Collected samples were achieved twice a week visits to the Dermatology outpatient clinic, Tanta University hospital, Tanta, Egypt. Specimen collection was carried out by scraping the skin and hair scalp and then each specimen was cultivated on Sabouraud's dextrose agar (SDA) medium, amended with chloramphenicol (0.5g/L) to inhibit bacterial growth. For each patient, much information was collected to analyze their circumstancing conditions such as: gender, age, residential conditions (type of house, water source and sewage disposal), medical history of patients (first time or recurrent infection), patient occupational career (House wives, Students, handy professionals, farmers or indoor employers).

Diagnosis of cutaneous infection, isolation and identification of fungi from infected patients

The samples were isolated from patients that were clinically examined and diagnosed as cutaneous fungal diseases. The surface of the affected area was first scrubbed with cotton swab moistened with 70% ethyl alcohol prior to sampling. Scalpels, glass slides and Petri-dishes were previously sterilized to be used in sampling. Each sample was divided into 3 replica, each specimen (skin or hair fragments or hair scalps or cuttings from fingernails or from toenails) was cultured on 2 sterile Petri dishes with sterile Sabouraud's dextrose agar (SDA) medium; the third plate had the same composition of (SDA) in addition to cycloheximide (0.5g/L) to minimize the development of most saprophytic fungi other than dermatophytes; then cultured plates were incubated for 4 days at 35°C for the first two dishes to observe yeast and filamentus fugal growth and for 21 days at 25°C for the third plate to observe the dermatophytic growth (El-Shanawany, 1993).

The isolated filamentous fungi were macro and microscopically examined to be identified according to the following references; Gilman (1959), Raper & Fennel (1965) and Moubasher (1993). The isolated fungi were also photographed

by light microscope with a magnitude of 400 X.

Also other isolated yeast-like fungi were identified by API 20C Aux technique (API system, Montalieu, France) that was based on 19 carbohydrate assimilation tests plus a negative control, read by assessing cupules for turbidity. The kit was used in accordance with the guidelines given by the manufacturer (Barnett et al., 1990); all identified isolated were stored at 4°C for further work.

Algae collection, extraction, biosynthesis and characterization of AgNPs

Two marine algae *Corallina officinalis* L. and *C. mediterranea* Areschoug were collected from the Mediterranean Sea Coast, Alexandria, Egypt (Photo 1). The samples were examined and identified morphologically, depending on macroscopic and microscopic features according to Aleem (1993); then each sample was thoroughly washed by distilled water, shade-dried and powdered, separately. Each sample was divided into 2.5g fractions; which were boiled in 100ml of sterile distilled water for 5min. The crude extract was passed through Whatman No.1 filter paper and the filtrates were stored at 4°C. Two and half grams of the collected marine algae powder (Photo 1) were extracted with 100ml distilled water and filtered. 90ml of 1mM $AgNO_3$ solution (E. Merck) were added to 10ml of algal extracts slowly with magnetic stirring for even coating of silver and incubated at room temperature for 48hr under shaking of 120rpm in dark for the reduction of Ag^+ ions. The reduction of pure Ag^+ ions was monitored by color changing from pale yellow to red in the case of *Corallina officinalis* L. and from faint brown to dark brown in the case of *C. mediterranea* Areschoug, as shown in Photo 1. Two control flasks were maintained throughout the experiments, one contained aqueous $AgNO_3$ solution and the other contained aqueous algal extract. This green synthesis of AgNPs was performed according to a modified method by Vivek et al. (2011).

Photo 1. Collection of the selected marine algae and biosynthesis of AgNPs by their aqueous extracts.

Characterization of the obtained bio-nanomaterial was carried out by four different analytical assays, namely; UV-Vis spectral analysis, Transmission electron microscope (TEM) as well as Energy-dispersive X-ray (EDX) microanalysis and Fourier transform infrared spectroscopy (FT-IR).

UV-Visible spectroscopy of biosynthesized Ag-NPs by the two selected marine algae

The reduction of silver ions Ag^{+} in aqueous extracts of marine algae and the formation of Ag-NPs was monitored by measuring the UV-Vis spectra as a function of bio-reduction time at a wavelength of 190- 1100nm (Cao, 2004). Excess solution was removed using blotting paper (Schaffer et al., 2009). That was carried out on Ultra violet-Visible spectroscopy, T80+UV/VIS Spectrophotometer at Central laboratory, Tanta University, Egypt.

Transmission electron microscope (TEM)

Characterization of the size, shape and the assembly of Ag-NPs were monitored by using Transmission electron microscopic (TEM) analysis (JEOL JEM-2100) at Electronic Microscope Unit, Faculty of Science, Alexandria University, Egypt. Samples of AgNPs suspensions were collected after synthesis and concentrated at 60°C to reduce the volume of the solution. The concentrated solution was then centrifuged at 18g for 15min. The pellets obtained were washed and re-dispersed in deionized water. This process was repeated to remove water-soluble biomolecules and to purify AgNP suspension, and then the suspension was ultra-sonicated for 2min. Two drops of each sample were placed onto carbon-coated TEM grids. Then the film on the TEM grid was dried prior to measurement. The average diameter of different 10 particles of AgNPs was calculated for each suspension (Eppler et al., 2000).

Energy dispersive X-ray spectroscopy (EDX)

Energy dispersive X-ray (EDX) spectroscopy was used to measure the purity of the formed AgNPs according to Strasser et al. (2010) at Electronic Microscope Unit, Faculty of Science, Alexandria University, Egypt.

Fourier-transform infrared (FTIR)

This assay was performed for both AgNPs extracts obtained from the two selected marine algae to detect the presence or absence of unsaturated aliphatic bonds (double or triple) and other toxic derivatives; and capping of AgNPs, by analyzing the samples separately using Bruker-Tensor-27-FT-IR spectrometer (Germany) at Central laboratory, Tanta University, Egypt. The FT-IR spectrum of the each AgNPs was obtained by determination of functional groups with the help of IR correlation charts. The IR spectra were reported in % transmittance and represented graphically as a relation between % transmittance versus wavenumber (cm^{-1}). AgNPs samples were prepared by compression of one AgNPs colloidal solution drop between two transparent KBr discs (Philip, 2009).

Estimation of antifungal activity of the biosynthesized silver nanoparticles against the selected cutaneous fungi in comparison with commercial antifungal agents

All collected fungal isolates from cutaneous mycosis patients were tested for susceptibility against the AgNPs biosynthesized by the two selected marine algae in the present survey, in comparison with two commercially available antifungal agents (itraconazole and miconazole, Jansen-Cilag, Belgium) by well diffusion method, described by Bauer et al. (1966). AgNPs suspensions were Lyophilized to obtain a dry powder for accurate for preparation of definite concentrations for each sample, this process was carried out at the Medical Researches Institute (Alexandria, Egypt). Sabouraud's dextrose agar (SDA) medium was prepared, sterilized, poured in sterile Petri dishes; then each plate was inoculated with 1ml of a pure suspension of fungal growth for different isolates obtained in the present cutaneous infections; and dispersed homogenously. Regular wells were made by sterile cork borer after the solidification of the inoculated medium. All powders of the tested AgNPs and commercial antifungal agents were suspended separately in sterile distilled water with concentration of 100mg/ml, and then 100µL of each suspension were dropped in a separate well in the pre-inoculated plates. For each fungal isolate, a plate with 4 wells was used to test its susceptibility against both 2 AgNPs and 2 commercial antifungal agents, and 3 plates were prepared as replica for each isolate. Plates were incubated for 4 days at 35°C to observe yeast and filamentous fugal growth and incubated for 21 days at 25°C to observe the dermatophytic growth.

Minimum inhibitory concentration (MIC) test was carried out to confirm the effective antifungal activity of Ag-NPs biosynthesized by the selected marine algae against the fungal growth of the most common and the most affected human pathogenic fungal isolates in the present study; that could be indicated by the least percentage of surviving cells with observably low concentration of AgNPs. MIC test was performed according to Baker et al. (2005); as liquid cultures were prepared by adding 9ml of liquid culture media + 0.5ml of spore or cell suspension with $6x10^6$ spore or cell/ml+ 0.5ml of AgNPs suspension of different bi-fold dilutions; 2, 1, 0.5, 0.25, 0.125 and 0mg/ml for AgNPs obtained from *Corallina officinalis* L. and 1, 0.5, 0.25, 0.125, 0.06 and 0mg/ml for AgNPs obtained from *C. mediterranea* Areschoug. Prepared mixtures were incubated at 35°C for 24hr. for *Candida tropicalis* and at 27°C for 72hr. for *Microsporum canis*, *Epidermophyton floccosum* and *Aspergillus ochraceous*. Then the percentages (%) of surviving cells were determined by spectrophotometer at 520nm for each sample and calculated as follows, then MIC was indicated graphically.

$$\% \text{ of surviving cells} = [(A_{0.0} - A_{conc.})/A_{0.0}] \times 100$$

while: $A_{0.0}$= The spectrophotometer reading of untreated sample (concentration of AgNPs= 0.0mg/ml).

$A_{conc.}$= The spectrophotometer reading of a certain concentration of AgNPs suspension.

Ultra-structural effects of AgNPs biosynthesized by the two selected marine algae on the most affected fungal isolates in the present survey

This assay was performed to study the effect of AgNPs biosynthesized by the two selected marine algae on the ultra-structure of the fungal isolates obtained from patients of cutaneous infections, that were observed to undergo the greatest antifungal activity of AgNPs in the present study, the selected fungal isolates were cultured on SDA liquid medium, and then mixed with AgNPs suspensions separately, which were obtained from the tested algal extracts and prepared with the previously recorded MIC. All the mixtures were incubated overnight on a shaking incubator of 60rpm at the appropriate temperature. Then treated mixture was centrifuged at 3000rpm for 20min, washed with sterile saline solution, re-centrifuged to collect the cell pellet in a clean Eppendorf tube (Richards & Cavill, 1976).

Collected cell pellets were fixed by adding 1ml of 2.5% glutaraldehyde, that was buffered in 0.1M phosphate buffer saline (PBS) of pH= 7.4 (for fixation of cellular protein content, and to stop all culture reactions), and cooled at 4°C for 2hr. Fixed samples were washed with 1% osmic acid for 30min (for fixation of lipid cell content), washed 3 times with PBS (10min for each time), and dehydrated in ascending ethanol concentrations (30, 50, 70, 90 and absolute alcohol) for 30min for each concentration, then dehydrated samples were infiltrated with acetone for 1hr; 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. Full-stained ultra-thin sections were examined, and photographed under the appropriate magnification, using the transmission electron microscope (JEOL-JEM-100SX, Japan) with beam current= 60µA and high voltage of 80KV (Ardenne & Beischer, 1940).

Results and Discussion

Survey of predisposing factors and the incidence of human cutaneous fungal infections during 2016

Residential conditions were summarized in Table 1 for the studied 80 patients, recording that the highest incidence of cutaneous fungal infections occurred in patients aged between 2-20 years old (38 cases), while other age categories over 20 years old possessed 42 cases and more common among females (47 cases) rather than male patients (33 cases). Patients who were living in rural houses (49 cases) suffered from cutaneous mycosis rather than patients in urban houses (31 cases). Indoor water source with closed pipe network (73 cases) and sewage disposal by outdoor conservancy (68 cases) were the most effective on the spread of cutaneous fungal infections, that was mostly recorded as a first time infection (54 cases) as recorded in Table 1. These data summarized the hazard role of different patient circumstances in facilitating the exposure to cutaneous fungal infections. It could be a new trend of patients' awareness for better hygienic behaviors to help in avoiding infection and to control the its spread from patients to healthy people.

TABLE 1. Survey of different residential conditions and other predisposing factors, affecting the incidence of human cutaneous fungal infections during 2016.

Predisposing factors	Most frequent category	No. of cases for each category	Other categories of lower effect	No. of cases
Age	Range of 2-20 years old	38	More than 20 years old	42
Gender	Female	47	Male	33
Residence	Rural houses	49	Urban houses	31
Water source	Closed pipe network	73	Outdoor pump	7
Sewage disposal	Outdoor conservancy	68	Closed pipe network	12
History of infection	First time infection	54	Recurrent infection	26

Table 2 recorded the incidence of cutaneous fungal infection among patients with different occupational careers. It was observed that the highest rate of infection was recorded for housewives (26 cases= 32.5 %), followed by students aged 5-18 years old (17 cases= 21.2 %), then farmers and handy professionals were represented in 15 and 14 cases (18.8 and 17.5%). While indoor employees recorded the lowest rate of infection (8 cases= 10%).

TABLE 2. Distribution of human cutaneous fungal infections among different occupational careers of the studied patients during 2016.

Occupation	No. of cases	% out of total cases
House wives	26	32.5
Students	17	21.2
Farmers	15	18.8
Handy professionals	14	17.5
Indoor employers	8	10
Total	80	100

These findings were correspondent to Woldeamanuel et al. (2005), who recorded that Tinea corporis is the most common dermatophyte infection among students, especially in the African population and supported by the statement of Moriarty et al. (2012), who reported that in the United States, Tinea capitis most commonly affected house wives and indoor workers. That was also in agreement with Lorch et al. (2010), who reported that Tinea unguium mainly affects farmers and handy professionals.

Diagnosis of cutaneous infection, isolation and identification of fungi from infected patients

Macroscopic and microscopic features of some representative fungal causative agents, isolated from different types of cutaneous fungal infections in the present survey were illustrated in Photo 2; in the meantime, the data recorded in Table 3 indicated that Tinea capitis possessed the highest incidence among patients (29 cases), caused by *Microsporum canis*, which was isolated from 21 cases and *Epidermophyton floccosum*, which was isolated from 8 cases. That was followed by Tinea unguium (23 cases), which was caused by different species of *Candida*; namely *C. albicans* (9 cases), *C. tropicalis* (7 cases) and *C. parapsilosis* (6 cases). While *Malassizia furfur* caused moderate incidence of Tinea versicolor among 16 cases. *Trichophyton rubrum* (11 cases= 13.75%). The lowest incidence was recorded for Tinea corporis (13 cases), that was caused by *Trichophyton rubrum* (11 cases) and *Aspergillus ochraceous* (2 cases). These findings were supported by Raccurt et al. (2009) who reported that the common causes for tinea corporis (ringworm) in Canada include *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Microsporum canis*. Also, Hainer (2003) reported that *T. rubrum* is the most common infectious agent in the world and is the source of 47% of tinea corporis cases, tinea pedis is most commonly caused by *Trichophyton rubrum* (60%), *T. mentagrophytes* (20%), *Epidermophyton floccosum* (10%); while more rarely by *Microsporum canis* and *T. tonsurans* and yeast such as *Candida* species were also found to be responsible for Tinea unguium. This was also supported by the results of Arenas (2015) which recorded that these common fungal infections represent 50% of total nail disorders and affect about 10% of the general population with frequencies that vary in different areas of the world. These types were usually observed in adults of urban areas, this record was confirmed by Arenas (2014) which recorded that adults are more likely to develop tinea cruris, tinea pedis, and tinea unguium (onychomycosis). One fungal

specie was rarely represented among the studied infections in this survey, namely *Aspergillus ochraceous*, which is traditionally a soil fungus, has now began to adapt to varied ecological niches, like agricultural commodities, farmed animal and marine species (Ostry et al., 2013); the pig and chicken populations in the farms are the most affected by this fungus and its mycotoxins (Stoev et al., 2010).

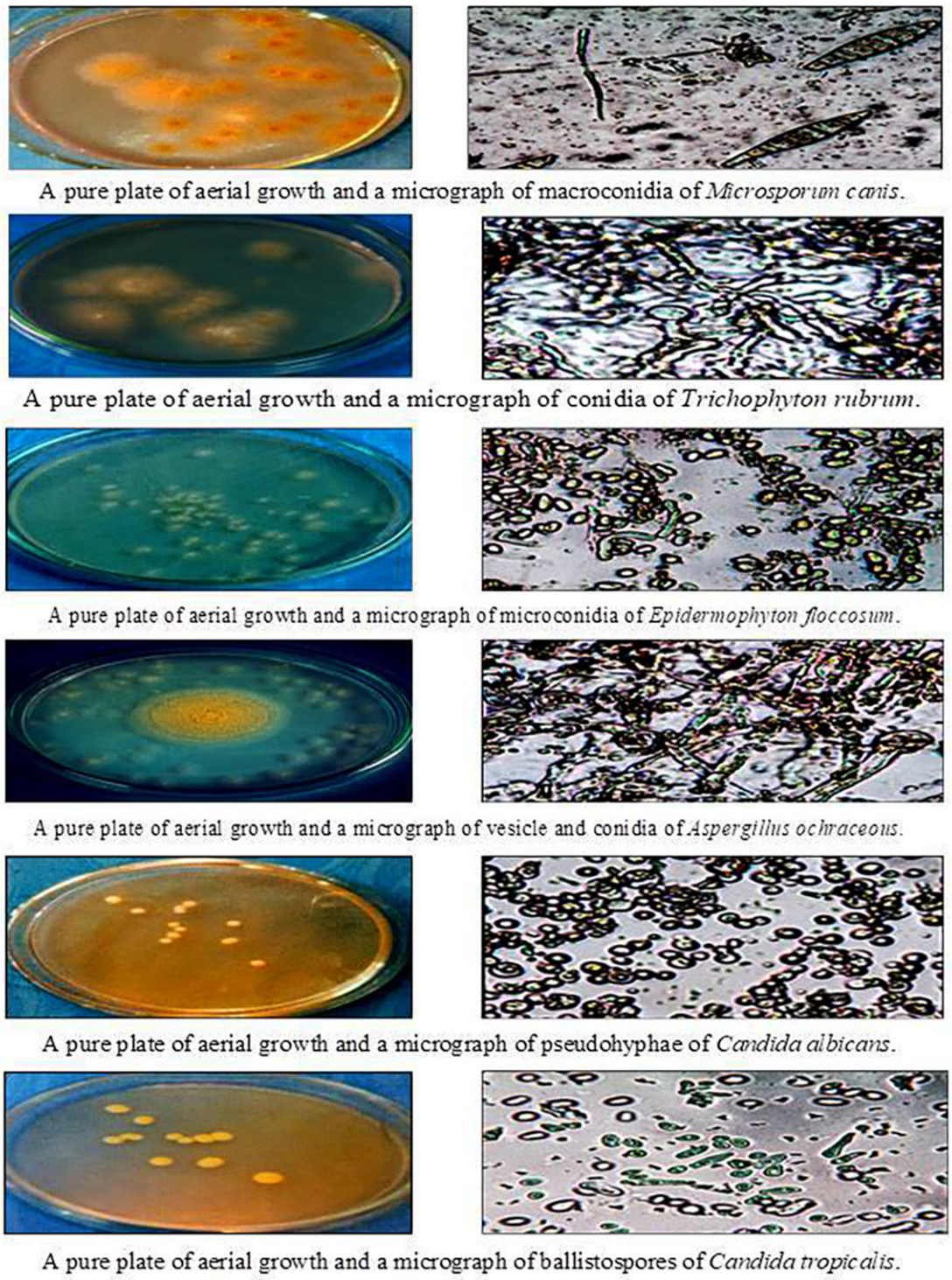


Photo 2. Culture and micrographs of some representative cutaneous fungal isolates in the present survey.

TABLE 3. Incidence of different types of Tinea and their causative fungi among the studied patients during 2016.

Types of Tinea	Fungal isolates	No. of cases	% out of total cases
	Yeast-like fungi:		
Tinea versicolor	*Malassizia furfur* (Sabouraud, 1904)	16	20
	Candida albicans (Robic, 1853)	9	11.2
Tinea unguium	*Candida tropicalis* (Castellani, 1910)	7	8.8
	Candida parapsilosis (Poll, 1926)	6	7.5
	Filamentous fungi:		
Tinea capitis	*Microsporum canis* (Bodin, 1902)	21	26.2
	Epidermophyton floccosum (Harz, 1870)	8	10
Tinea corporis	*Trichophyton rubrum* (Malmsten, 1845)	11	13.8
	Aspergillus ochraceous (Traboschi, 1908)	2	2.5
Total		80	100

Characterization of silver nanoparticles (AgNPs) biosynthesized by the selected marine algae

The formation of AgNPs was observed by measuring the change of color of the silver-algae extract solution. The color was changed from pale yellow to red in case of *Corallina officinalis* L. and from faint brown to dark brown in case of *Corallina mediterranea* Areschoug, indicating the synthesis of AgNPs as illustrated previously in Photo 1. The color change of the solution is due the Surface Plasmon Resonance (SPR) phenomenon. Where purity, shape, size, aggregation and toxic impurities were confirmed by the following analytical assays:

Scanning absorbance of UV-Visible spectroscopy versus wave length (λ) was shown in Figs. 1 and 2. The characteristics peaks of silver nanoparticles which were due to charge transfer spectra the two absorption peaks at wave lengths of 448nm and 451nm indicated the formation of Ag-NPs by *Corallina officinalis* L and *C. mediterranea* Areschoug.

The shape and size of Ag-NPs biosynthesized by the selected two marine algal extracts were confirmed by Transmission Electron Microscope (TEM), as illustrated in Photos 3 and 4. Table 4 recorded the average size of biosynthesized Ag-NPs for *Corallina officinalis* L. and *Corallina mediterranea* Areschoug (9.64nm and 8.29nm) with a cubic regular shape. Also, it could be observed that the formed AgNPs had no aggregations.

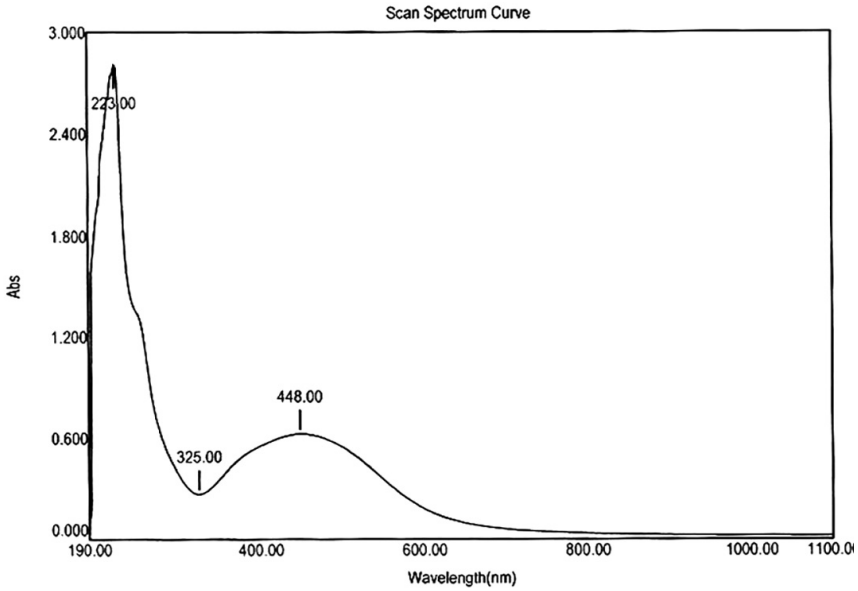


Fig. 1. UV-Visible absorption spectra of AgNPs biosynthesized by the marine alga *Corallina officinalis* L.

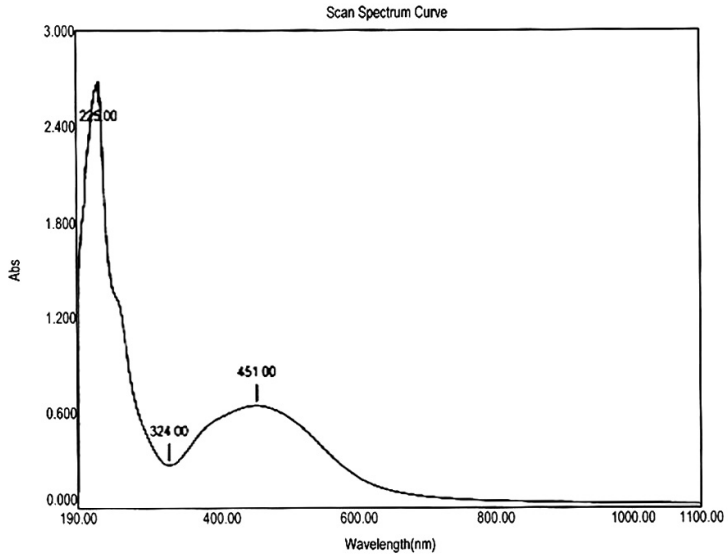


Fig. 2. UV-Visible absorption spectra of AgNPs biosynthesized by the marine alga *Corallina mediterranea* Areschoug.

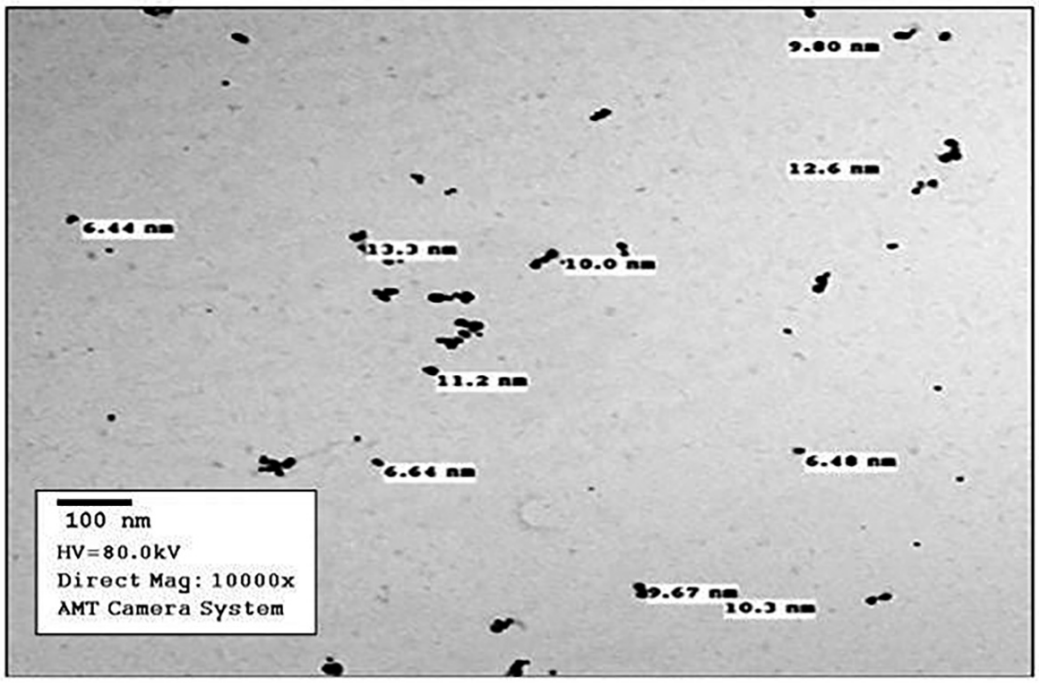


Photo 3. Transmission electron micrograph of AgNPs, biosynthesized by the algal aqueous extract *Corallina officinalis* L..

Figures 3 and 4 illustrated the Energy Dispersive X-ray Spectroscopy (EDX), in which the purity percentage is 63.1% for washed AgNPs biosynthesized by *Corallina officinalis* L. and 87.5% for AgNPs biosynthesized by *C. mediterranea* Areschoug; with appearance of many small peaks, indicating the presence of other normally present nutrient elements with rare amounts.

Fourier-transform infrared (FT-IR) spectrum were illustrated in Figs. 5 and 6 for AgNPs bio synthesized by *Corallina officinalis* and *C. mediterranea*, revealing the incidence of saturated aliphatic bonds and atomic groups of normally present carbohydrates and proteins and confirming the absence of toxic triple bonds and cyanide groups.

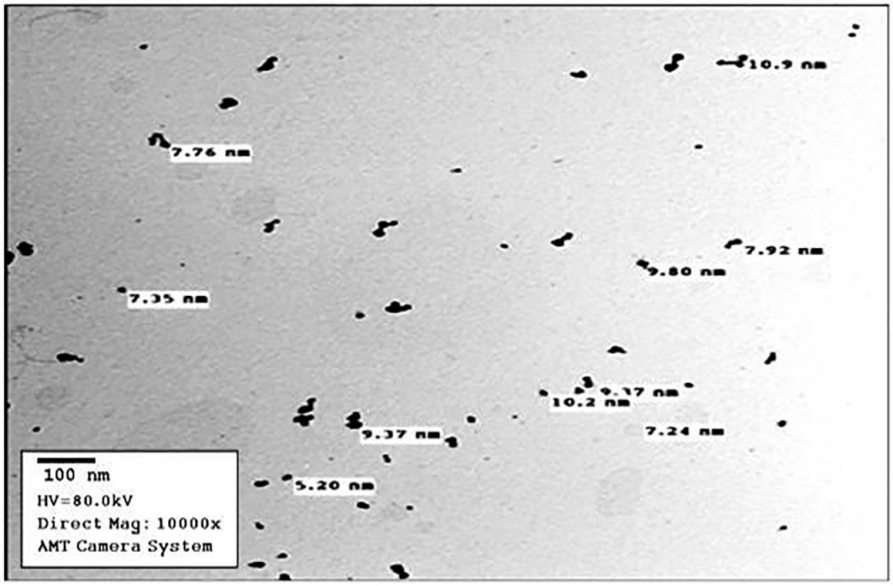


Photo 4. Transmission electron micrograph of AgNPs, biosynthesized by the algal aqueous *C. mediterranea* Areschoug

TABLE 4. Mean size of AgNPs biosynthesized by the both selected marine algae, confirmed by TEM measurement.

	Diameter of AgNPs (nm)	
	Aqueous extract of *Corallina officinalis*	**Aqueous extract of *C. mediterranea***
Reading replica	6.44	7.76
	13.30	10.90
	9.80	7.35
	12.60	9.37
	10.00	5.20
	11.20	7.92
	6.64	7.80
	6.48	9.17
	9.67	10.20
	10.30	7.24
Mean value	9.64	8.29

Characterization tests performed in the present study, confirmed the small size of AgNPs, high purity of their biologically prepared extracts and the absence of toxic derivatives. These findings in agreement with Jain et al. (2009) and Ahmed et al. (2010), who stated that marine algae contain several biologically active molecules which are used as source of food, feed and medicine; as more than 2400 marine natural products have been isolated from seaweeds; also, it was well known that the cell wall of seaweeds contain many functional molecules like amine, carboxyl, sulphate, phosphate and imidazoles associated with polysacchride, alginic acid and proteins which enable them to bind with silver ions to reduce them into silver nanoparticles (Dibrov et al., 2015). Small size of AgNPs under miceroscope indicated high stability of AgNPs, high penetrative and absorptive effeciency and maintenance of their properties. These findings were supported by Dibrov et al. (2015) who recorded the characters for silver nanoparticles biosynthesized by *Ulva lactuca*. EDX FT-IR profile data confirmed the high productivity and purity of AgNPs obtained by the two selected marine algae in the aqueous medium; that was supported by the work of Magudapathy et al. (2001) and Awwad et al. (2013).

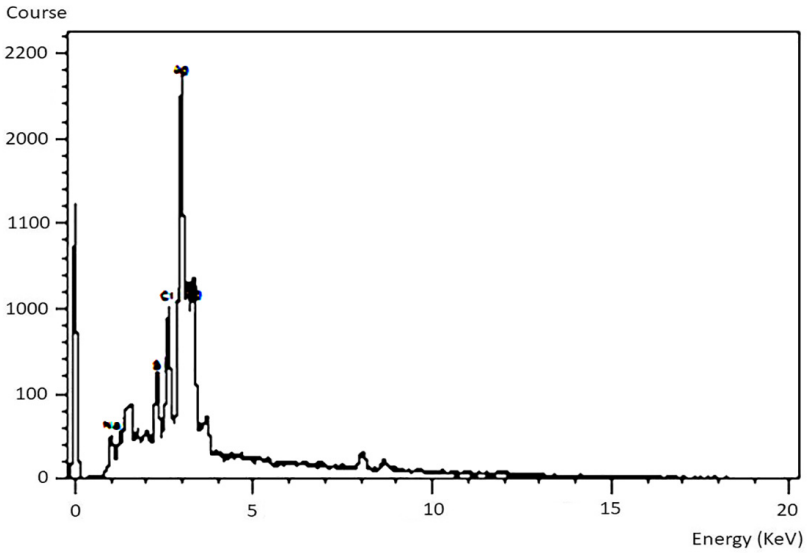


Fig. 3. Energy-dispersive X-ray analysis purity profile of AgNPs biosynthesized by *Corallina officinalis* L.

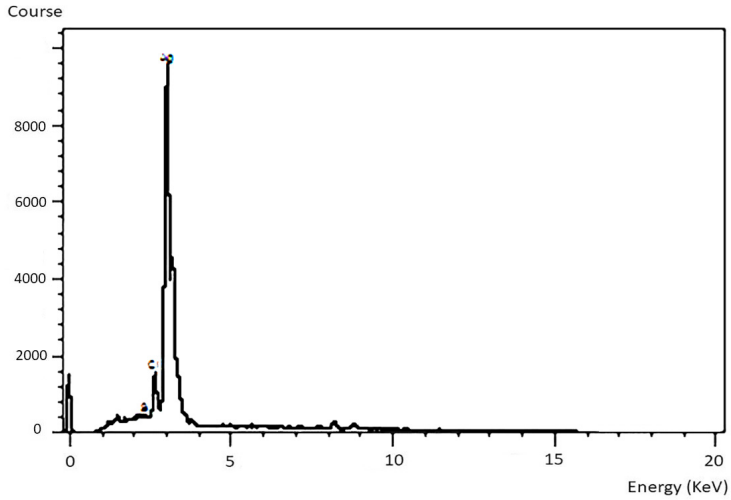


Fig. 4. Energy-dispersive X-ray analysis purity profile of AgNPs biosynthesized by *Corallina mediterranea* Areschoug.

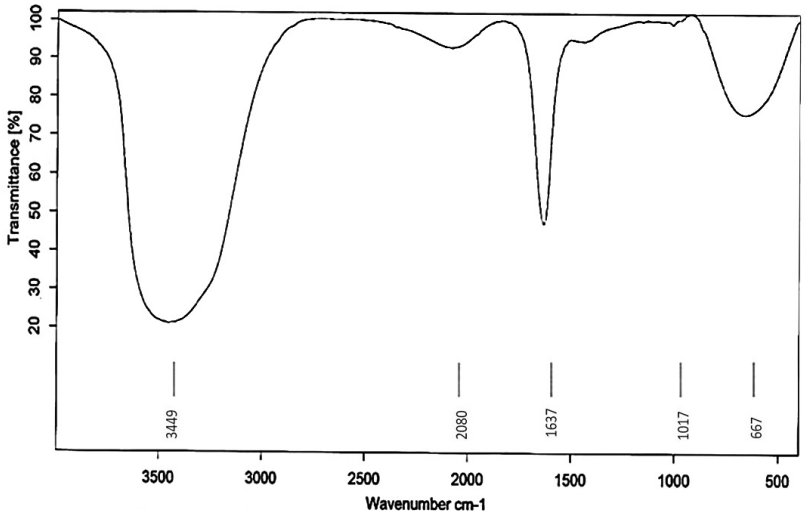


Fig. 5. Fourier-transform infrared spectrophotometric profile of AgNPs biosynthesized by marine alga *Corallina officinalis* L.

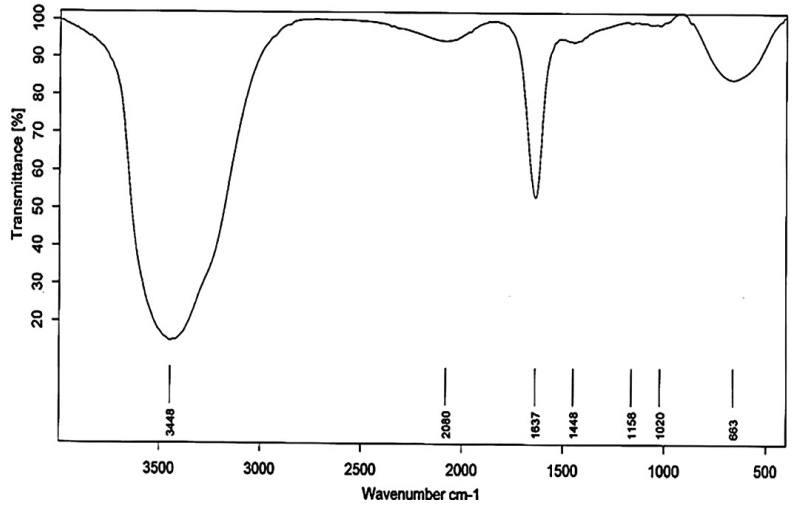


Fig. 6. Fourier-transform infrared spectrophotometric profile of AgNPs biosynthesized by marine alga *Corallina mediterranea* Areschoug.

Estimation of antifungal activity of the biosynthesized silver nanoparticles against the selected cutaneous fungi in comparison with commercial antifungal agents

From Table 5, it was observed that Ag-NPs biosynthesized by *Corallina officinalis* had the most effective inhibitory action against *Candida tropicalis*, with an inhibition zone diameter of 22.6mm, while it possessed less effect against *Microsporum canis,* with an inhibition zone diameter of 14.6mm; in the meantime, Ag-NPs biosynthesized by *C. mediterranea* showed moderate inhibitory action against *Epidermophyton floccosum* with an inhibition zone diameter of 16.6 mm, while it possessed less effect against *Aspergillus ochraceous,* with an inhibition zone diameter of 15.6mm; compared with lower effect of commercial itraconazole and miconazole, recording inhibition zone diameters of 13.3mm and 18.3mm against *Candida tropicalis*, 10.6mm and 11.3mm against *Microsporum canis*, 10.7mm and 12.6mm against *Epidermophyton floccosum* and 11.6mm and 13.6mm against *Aspergillus ochraceous*; as all agents were prepared with unified concentrations, as recommended for the traditionally available antifungal use.

The records represented in Figs. 7 and 8 indicated that MIC of AgNPs biosynthesized by *Corallina officinalis* was 0.5mg/ml against *Candida tropicalis* and *Microsporum canis*, while MIC of AgNPs biosynthesized by *C. mediterranea* was 0.25mg/ml against *Epidermophyton floccosum* and *Aspergillus ochraceous*.

The effective MIC values recorded for AgNPs in the present survey was correlated by Fatima et al. (2016), who stated that *A. flavus* and *A. niger* recorded 17mm and 15mm of inhibition zone diameter with as low as 5mg/ml of AgNPs suspension. Also, Petica et al. (2008) explained the high inhibitory activity of silver nanoparticles against fungi by the interaction with cell proteins, leading to the inactivation of fungal growth and direct interaction with DNA resulted in inhibition of replication.

TABLE 5. Preliminary survey for antifungal activity of AgNPs biosynthesized by the two selected marine algae, against the most affected fungal isolates, obtained from human cutaneous infections in the present study, compared with commercial antifungal agents.

Antifungal agent	Mean diameter of inhibition zone (mm)			
	Candida tropicalis	***Microsporum canis***	***Epidermophyton floccosum***	***Aspergillus ochraceous***
Itraconazole	13.3±0.9	10.6±0.8	10.7±0.8	11.6±0.9
Miconazole	18.3±1.1	11.3±1.2	12.6±0.9	13.6±0.6
AgNPs of *C. officinalis*	22.6±1.4	14.6±0.9	--	--
AgNPs of *C. mediterranea*	--	--	16.6±0.8	15.6±0.9

Each value= Mean of 3 replica± standard deviation.

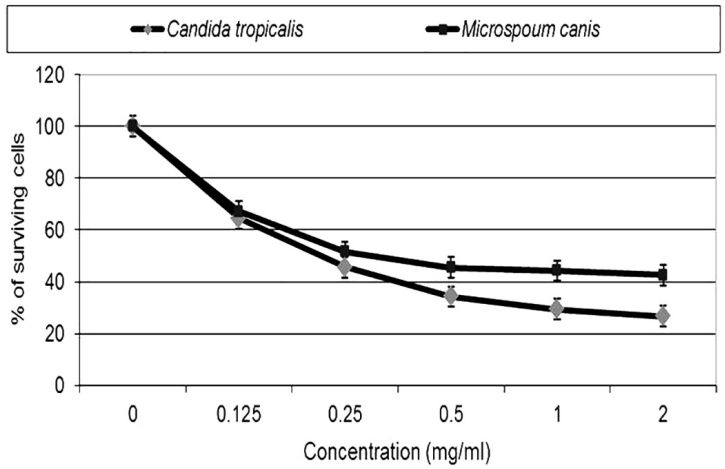


Fig. 7. Estimation of MIC of AgNPs biosynthesized by *Corallina officinalis,* by affecting the surviving ratios of *Candida tropicalis* and *Microsporum canis*.

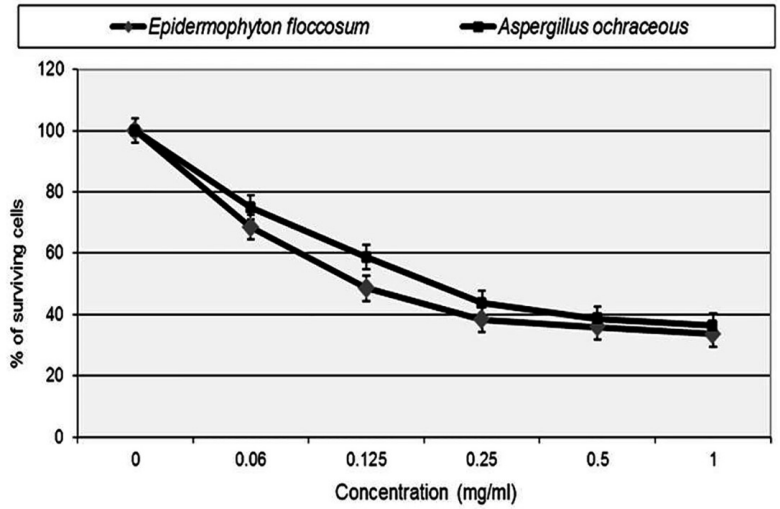


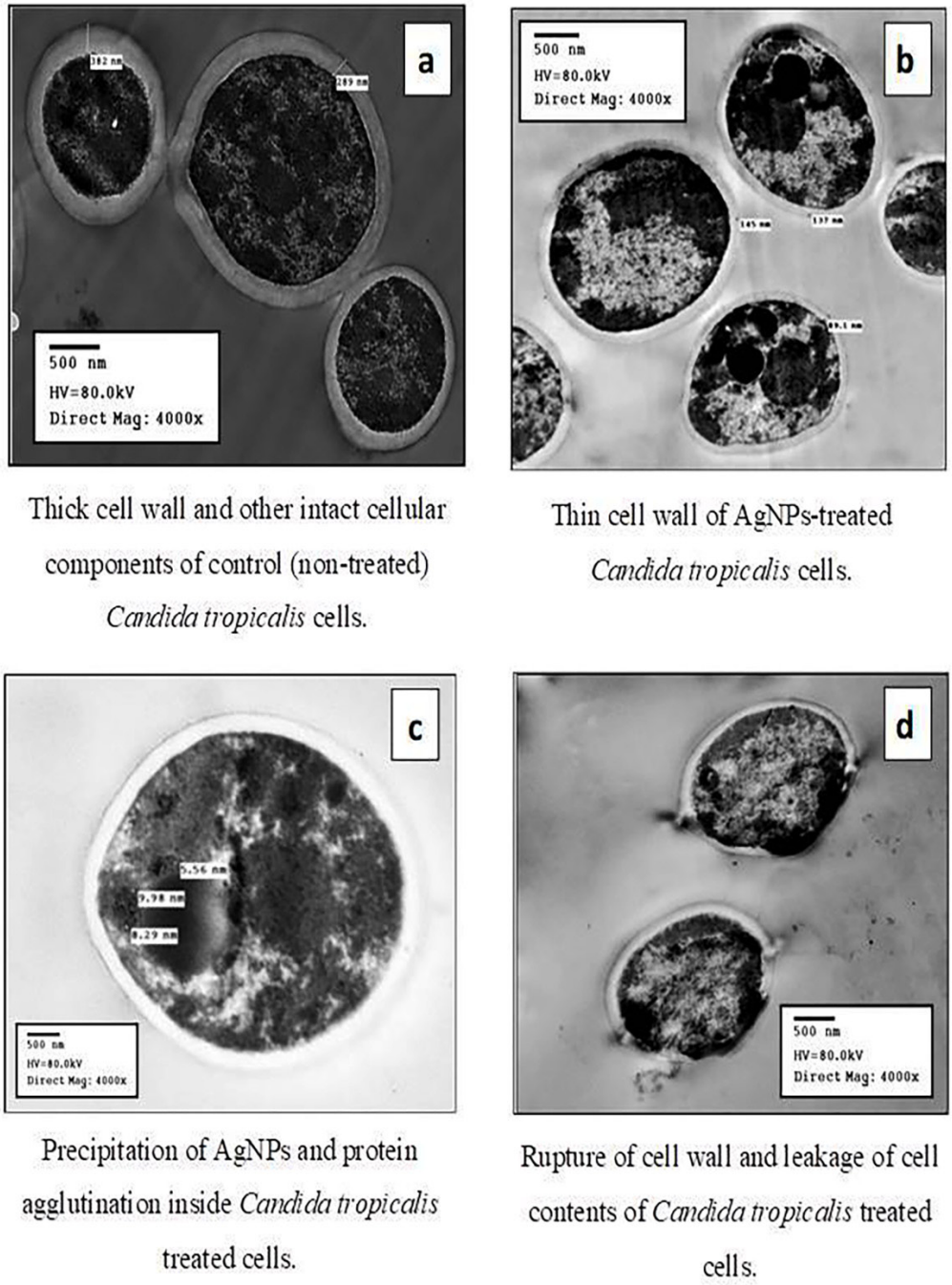
Fig. 8. Estimation of MIC of AgNPs biosynthesized by *Corallina mediterranea,* by affecting the surviving ratios of *Epidermophyton floccosum* and *Aspergillus ochraceous.*

The mechanism of antifungal activity of silver nanoparticles was not fully known. A few of the literature illustrated the possible mechanism of antifungal activity. Spherical shape and small size of reduced silver ions caused the increased contact area so that it ensured the inhibition of fungal growth; so small silver nanoparticles had similar effects as silver ions (Bai et al., 2011); positive charged silver ions may attach with negatively charged cell membranes of fungi by electrostatic attraction (Mohan et al., 2007). Ag-NPs could attach to cell membrane and penetrated into the fungal cell, and then AgNPs could attach to respiratory sequence and finally cell division stop, leading to cell death (Lewis, 2004). Monteiro et al. (2012) concluded that silver nanoparticles biosynthesized by marine macro algae had a great potential to controll the spore production by fungi.

Noorbakhsh et al. (2011) confirmed that the antifungal activities of fluconazole and griseofulvin were increased in the presence of AgNPs. Ability of AgNPs in destroying fungi was suggested to be due to perforation of cell wall and plasma membrane (Rai, 2009). Kim et al. (2008) showed that AgNPs had inhibitory effects on the growth of *T. mentagrophytes*, *C. albicans*, *C. tropicalis* and *C. glabrata*. AgNPs (1-7µg/ml). Also, Teimoori et al. (2017) recorded low resistance of *A. flavus* against AgNPs as effective antifungal agent. In the meantime, El-Kadi et al. (2018) proved the higher antifungal activity of AgNPs than ordinary $AgNO_3$ solution, as they reported up to 83.11% growth inhibition for *Aspergillus flavus, A. ochraceus, A. glaucus, A. niger* and *Penicillium* sp.

Ultra-structural effects of AgNPs biosynthesized by the two selected marine algae on the most affected fungal isolates in the present survey

Photo 5 showed ultra-structural effects of AgNPs biosynthesized by *Corallina officinalis* on *Candida tropicalis*. Photo 5-a recorded the normal thickness (382- 389nm) of cell wall and intact internal cellular structures of control (non-treated) growth of *Candida tropicalis*. While Photo 5-b illustrated the thinning of cell wall (89.1- 145nm) of AgNPs-treated cells of *C. tropicalis*; then Photo 5-c revealed the precipitation of AgNPs inside the cytoplasm and agglutination of intracellular proteins and Photo 5-d showed the rupture of cell membrane and the leakage of cellular components outside the AgNPs-treated cells of *C. tropicalis*.



Thick cell wall and other intact cellular components of control (non-treated) *Candida tropicalis* cells.

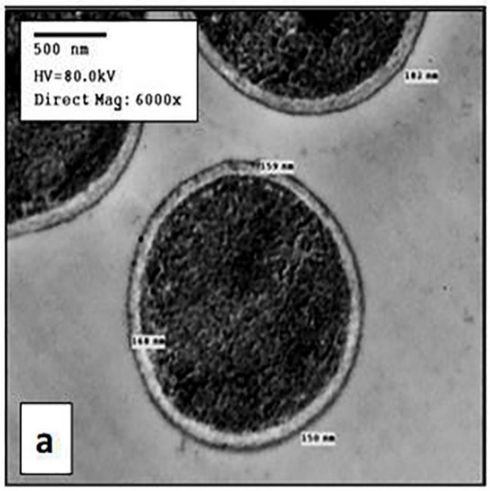
Thin cell wall of AgNPs-treated *Candida tropicalis* cells.

Precipitation of AgNPs and protein agglutination inside *Candida tropicalis* treated cells.

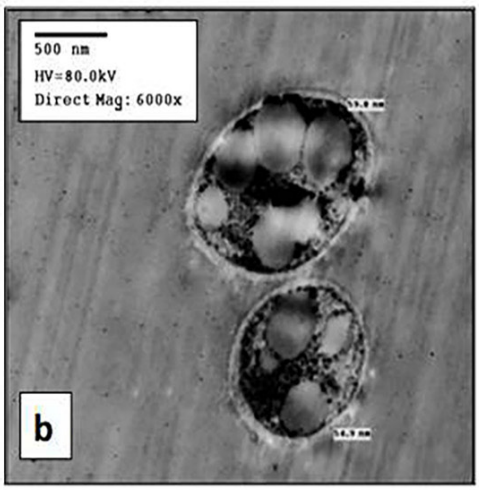
Rupture of cell wall and leakage of cell contents of *Candida tropicalis* treated cells.

Photo 5. Transmission electron micrograph of AgNPs, biosynthesized by *Corallina officinalis* affecting cell wall and intracellular structures of *Candida tropicalis*.

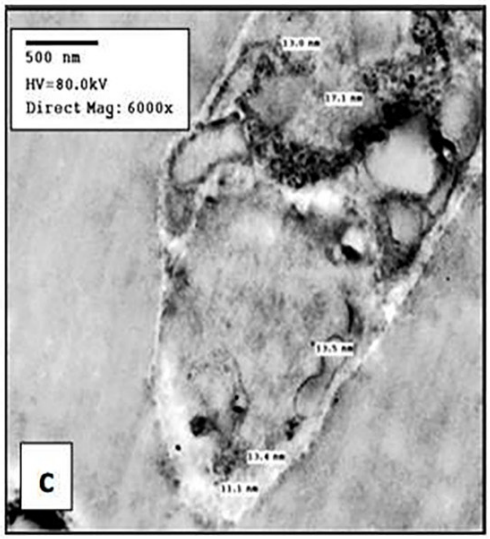
Photo 6 showed ultra-structural effects of AgNPs biosynthesized by *C. mediterranea* on *Epidermophyton floccosum*. Photo 6-a recorded the normal thickness (150- 182nm) of spore wall and intact internal structures of control (non-treated) growth of *E. floccosum*. While Photo 6-b illustrated the thinning of spore wall (54.9-59.8nm) of AgNPs-treated cells of *E. floccosum*; then Photo 6-c revealed the precipitation of AgNPs inside the cytoplasm and agglutination of intracellular proteins; and Photo 6-d showed the rupture of cell membrane and the leakage of cellular components outside the AgNPs-treated hyphae of *E. floccosum*.



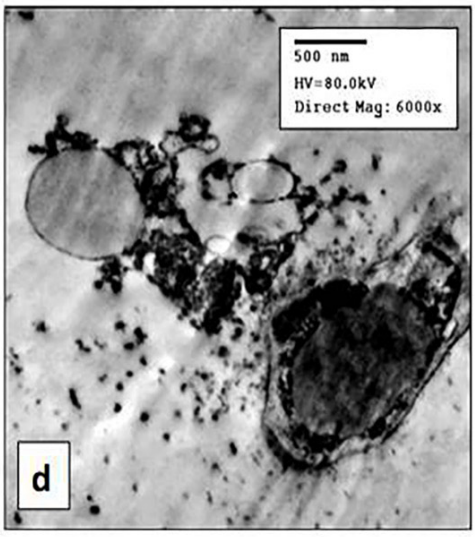
Thick wall and other intact cellular components of control (non-treated) *Epidermophyton floccosum* conidia.



Thin wall of AgNPs-treated *Epidermophyton floccosum* conidia.



Precipitation of AgNPs and protein agglutination inside *Epidermophyton floccosum* treaed hyphae.



Rupture of cell wall and leakage of cellular contents of *Epidermophyton floccosum* treaed hyphae.

Photo 6. Transmission electron micrograph of AgNPs, biosynthesized by *Corallina mediterranea* affecting cell wall and intracellular structures of *Epidermophyton floccosum*.

These findings indicated that the biosynthesized AgNPs had a great inhibitory and destructive effect on the growth of the tested cutaneous fungal isolates. Kim et al. (2009), Hwang et al. (2012) and Vazquez et al. (2014) explained the antifungal activity of AgNPs against *Candida albicans* and reported that the disturbance in membrane potential and creating pores therefore provoked the ion leakage and other components, leading to cell lysis and death. The interaction of nanoparticles with cell surface leads to disruption of the outer cell wall and subsequently produce permealization of cell membrane that let smaller AgNPs to get inside the cell with much more amounts, that is supported by Swathy (2014) who recorded that AgNPs exerted an activity against fungi mainly via the permealization of target cells.

Conclusion

The most common fungal isolates among the studied cutaneous infections were *Microsporum canis, Malassezia furfur, Trichophyton rubrum, Candida albicans, Epidermophyton floccosum* and *Candida tropicalis*, which were more common in patients depended on indoor closed pipe network as a water source, sewage disposal by outdoor conservancy, house wives and age of 2- 20 years old. AgNPs biosynthesized by both extracts of marine algae *Corallina officinalis* and *C. mediterranea* were characterized their small size, definite cuboid shape, high concentration of the produced colloidal suspension with observable purity and absence of other toxic impurities.

Low value of MIC recorded for AgNPs obtained by the selected marine algae, indicated their high antifungal activity against *Epidermophyton floccosum* and *Candida tropicalis*. That was confirmed by the destructive effects, as observable thinning of cell wall, agglutination of cellular proteins, rupture of cell membrane and leakage of intracellular components were revealed through TEM examinations.

Recommendation: The outputs of the present survey recommend more focus on patient education about health awareness and avoiding the polluted water sources. Also, more studies should deal with the use of AgNPs biosynthesized by naturally available macroalgae as a new remedy against cutaneous fungi to avoid the side effects of traditional antifungal agents.

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علاج جديد للعدوى الفطرية لجلد الإنسان باستخدام جزيئات الفضة متناهية الصغر المخلقة حيوياً بواسطة إثنان من الطحالب البحرية الكبيرة

أنور البدرى[1]، سوزان السواح[1]، هالة القصاص[2]، دعاء حجاب[3] و داليا عامر[1]

[1]قسم النبات - كلية العلوم - جامعة طنطا - طنطا - مصر، [2]المعهد القومى لعلوم البحار والمصايد - الإسكندرية- مصر، [3] قسم الأمراض الجلدية- كلية الطب - جامعة طنطا - طنطا - مصر.

لقد تم دراسة حالات العدوى الفطرية المترددة على قسم الأمراض الجلدية بمستشفيات جامعة طنطا خلال عام 2016، و لوحظ أن العدوى الفطرية لجلد الإنسان كانت أكثر إنتشاراً بين المرضى مستخدمى شبكات مياه الشرب المنزلية المغلقة (73 حالة)، و الصرف الصحى الخارجى بالخزانات (68 حالة)، ذوى الأعمار ما بين 20-2 عاماً (38 حالة)، و ربات المنزل (23 حالة). و كانت أكثر المسببات الفطرية شيوعاً بين حالات العدوى الجلدية هى ميكروسبورام كانيس (21 حالة)، ملاسيزيا فيرفير (16 حالة)، ترايكوفايتون رابرام (11 حالة)، كانديدا ألبيكانز (9 حالات)، إيبيديرموفايتون فلوكوزام (8 حالات)، و كانديدا تروبيكاليس (7 حالات)؛ والذين تسببوا فى ظهور التينيا كابيتيس فى 29 حالة، التينيا أنجيوم (23 حالة)، تينيا فيرزيكولور (16 حالة)، و تينيا كوربوريس (13 حالة).

وقد سجلت الدراسة الحالية قدرة إثنين من مستخلصات الطحالب البحرية على تخليق جزيئات الفضة متناهية الصغر حيوياً، الذان أعطيا نشاط واضح مضاد للفطريات ضد العزلات الأكثر شيوعاً بين المرضى. و قد تأكد هذا النشاط ضد الفطرى بتسجيل قيم متدنية للتركيز المثبط الأدنى لكل منهما، حيث سجلت جزيئات الفضة متناهية الصغر المخلقة بواسطة مستخلص طحلب الكورالينا ميديتيرينيا تركيز مثبط أدنى 0.25 مجم/مل ضد فطر الإيبيديرموفايتون فلوكوزام، بينما سجلت جزيئات الفضة متناهية الضغر المخلقة بواسطة مستخلص طحلب الكورالينا أوفيشينياليس تركيز مثبط أدنى 0.5 مجم/مل ضد فطر الكانديدا تروبيكاليس. وقد أثبت هذا النشاط المضاد للفطريات بفحص التأثير المدمر لجزيئات الفضة متناهية الصغر على نمو الفطريات بواسطة المجهر الإلكترونى النافذ، حيث لوحظ تناقص سمك الجدار الخلوى فى الإيبيديرموفايتون فلوكوزام إلى 54.9 نانومتر وفى الكانديدا تروبيكاليس إلى 89.1 نانومتر، كما لوحظ إنتشار تجلطات بروتينية داخل السيتوبلازم، وتمزق الغشاء البلازمى للخلايا، مما أدى إلى فقدان المحتويات الداخلية لخلايا وخيوط وجراثيم الفطريات المعالجة.