ANTIFUNGAL EFFECT OF GOLD NANOPARTICLES ON FUNGI ISOLATED FROM ONYCHOMYCOSIS PATIENTS

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ABSTRACT

Onychomycosis is a widely distributed fungal nail infection which can be caused by dermatophytes, yeasts or non-dermatophytic fungi. In our study a total of 7 species related to 4 genera were isolated from 50 patients suspected with the fungal nail infection (onychomycosis) these namely *Aspergillus flavus* (8 isolates), *Aspergillus niger* (13 isolates), *Candida albicans* (8 isolates), *C. tropicalis* (5 isolates), *Epidermophyton floccosum* (1 isolate), *Trichophyton mentagrophytes* (2 isolates) and *Trichophyton rubrum* (1 isolate). Nowadays the field of nanotechnology becomes one of the most topics of interest. The impact of gold nanoparticles on the isolated fungal species was evaluated by agar well diffusion method and micro-dilution method. Best antifungal activity of gold nanoparticles was observed by using 100 µl of AuNps containing 20 µg of gold nanoparticles with the greatest zone of inhibition (19 mm) against *C. albicans*. AuNps showed variable MIC (minimum inhibitory concentration). MIC_{50} and MIC_{90} values of AuNPs ranged from 3.125 to 25.0 µg/ml and from 12.5 to100 µg/ml respectively. All isolated fungi could grow on keratin agar medium but with variable degrees indicating their ability to hydrolyze keratin. Keratinase activity in presence and absence of AuNPs was determined for dermatophytes and *Aspergillus* species. AuNPs had an inhibitory effect causing reduction in keratinase enzyme activity reaching 52.17 %, 40 % and 37.5 % was attained in case of *E. floccosum*, *A. flavus* and *A. niger* (the most susceptible isolates) respectively, by application of 20 µg/ml of AuNPs.

Keywords: Onychomycosis, Fungal infection, Gold, Nanoparticles, Keratinase.

INTRODUCTION

Onychomycosis is a superficial fungal infection, of the human nails, that is wide distributed worldwide leading to discoloration, nail plate thickening, and onycholysis (Adams *et al*., 2015; Soltani *et al*., 2015). Onychomycosis is used to describe fungal infection of one or more of the nail units and can be caused by dermatophytes (e.g. *Epidermophyton*, *Microsporum* and *Trichophyton* species), yeasts (e.g. *Candida* species) and non-dermatophytic fungi (*Aspergillus* species) (Weitzman and Summerbell, 1995; Kaur *et al*., 2008; Babayani *et al*., 2018). It is important to consider the specific causative organism when treating onychomycosis, because some organisms are less likely to respond to certain antifungal agents. Gold nanoparticles (GNPs) have attracted a great deal of interest and are the subject of intensive studies in biology and medicine owing to their extraordinary physicochemical properties, such as atmospheric stability, resistance to oxidation, surface functionalization, and biocompatibility (Loomba and Scarabelli, 2013).

Gold nanoparticles (AuNPs) generally are considered to be biologically inert but can be engineered to possess chemical or photothermal functionality (Li *et al*., 2014). The efficacy of the antibacterial activity of gold nanoparticles can be increased by adding antibiotics (Grace and Pandian, 2007; Bhattacharya *et al*., 2012). The gold nanoparticles generate holes in the cell wall, resulting in the leakage of cell contents and cell death. It is also possible that gold nanoparticles bind to the DNA of bacteria and inhibit the uncoiling and transcription of DNA (Rai *et al*., 2010; Rudramurthy *et al*., 2016). Although the synthesis of GNPs is very easy, the use of toxic chemicals in the synthesis of specific shape and size requirements limits their applications in biology (Murphy *et al*., 2008; Dykman and Khlebtsov, 2012). Therefore, the present study was designed to evaluate the impact of gold nanoparticles on the dermatophytic and non-dermatophytic fungi isolated from cases of onychomycosis as well as on their keratinase activity.

Materials and methods

Test organisms:

13 *Candida*, 21 *Aspergillus* and 4 dermatophyte fungal isolates were isolated from 50 patients suspected with fungal nail infection (onychomycosis) after obtaining approval of the Dermatology Research Ethical Committee of the Faculty of Medicine, Cairo University, study was performed during June 2015 to May 2016. The isolated fungi were identified on the basis of fungal identification. All *Aspergillus* and dermatophyte isolates were stored in Sabouraud's Dextrose Broth (OXOID) with 20 % glycerol at 80 ºC and propagated on Sabouraud's Dextrose Agar (OXOID) plates and incubated at 28 ºC before each test. The inocula were prepared from 7-day-old cultures. For *Candida* isolates, they were incubated at 37 ºC for 24 hours then one colony of each isolate was added to 20 ml of SDB and incubated at 37 ºC for 24 hours.

Preparation of gold nanoparticles (AUNPs)

Gold nanoparticles have been prepared and characterized at Nanotech company, Dream Land, Cairo, Egypt, by citrate reduction method as described by Turkevich *et al*. (1951).

Evaluation of antifungal activity of AUNPs

Antifungal activity of gold nanoparticles was carried out against the fungal isolates by Agar well-diffusion method (as a qualitative method) and minimum inhibitory concentration (MIC) (as a quantitative method). Suspensions of fungal isolates were freshly prepared by inoculating fresh stock from each isolate into separate Sabouraud's Dextrose Broth. The inoculated tubes were incubated at 28 ºC for 24 h. Fluconazole was used as an antifungal reference drug.

Agar well-diffusion method

The antifungal activity of gold nanoparticles against the tested fungal isolates was examined using agar well diffusion method. A 100 µl spore suspension of each tested isolate in SDB was spread individually by using sterile glass spreader on the surface of agar plates (9 cm diam) containing SDA. Wells (5-mm diameter), in the agar medium, were made using sterile cork poorer. Different volumes of AuNPs (5, 10, 30, 50, 100 µl) containing (1, 2, 6, 10, 20 µg) of AuNPs respectively were poured into each well. Fluconazole was used as antifungal reference drug. The plates were incubated at 28 ºC for 24 hours and the results were recorded by measuring the diameter of inhibition zone (mm) compared with the control (Magaldi *et al*., 2004).

Determination of MIC of AuNPs

The MIC for the different clinical isolates of fungi was determined by broth micro-dilution assay in accordance with the guidelines of the National Committee for Clinical Laboratory Standards as defined in document M38-A for filamentous fungi (CLSI, 2002). Suspensions of the test fungi were prepared by covering the fungal colonies, previously grown on 9-cm plates containing SDA for 7 days at 28 ºC, with 10 ml distilled water and then scraping the colony surfaces with the tip of a sterile loop. The resulting suspension containing conidia and hyphal fragments was transferred to sterile tubes and allowed to stand for 15 min at room temperature to allow the heavy particles and fragments to settle. The suspension was adjusted using a double-beam UV–visible spectrophotometer (model UV 2150; UNI CO) to 75 % to 80 % transmittance.

Preparation of native feather keratin powder

Native feather keratin powder was prepared according to the method of (Friedrich *et al*., 1999) from native chicken feathers.

Growth of fungal isolates on keratin medium

All fungal isolates were screened for their ability to digest keratin on keratin agar plates. Fungal inocula taken from 7-day-old cultures were inoculated on plates containing keratin agar medium which was prepared according to Friedrich *et al*. (1999). Three replicate plates were used for each isolate. The inoculated plates were incubated for 7 days at 25 °C and the diameters of clear zones were measured. Mean values were taken as criteria for keratinolytic activity for the tested fungal isolates.

Preparation of soluble keratin

Soluble keratin (SK) was prepared according to the method of (Wawrzkiewicz *et al*., 1987) with some modifications where native chicken feathers powder (10g) was solubilized in 500 ml of dimethyl sulfoxide (DMSO) with heating in a reflux condenser at 100 °C for 2 hours. Soluble keratin was then precipitated from the solution by the addition of cold acetone (1litre) at 20 °C for 2 hours. This step was then followed by centrifugation at 5000 rpm for 20 min (Hermle z230A). The precipitate was then washed twice with distilled water and finally dried at 50 °C.

Keratinase production

Erlenmeyer conical flasks (500 ml) each containing 100 ml of the keratinase production medium has the composition of (g/l): keratin, 10; glucose, 1.0 and distilled water, 1000 ml, were inoculated by spore suspension (1 x 10^6 spore/ml) from fungal isolates that have the greatest ability to degrade keratin on keratin agar medium namely *Aspergillus flavus, Aspergillus niger, Epidermophyton floccosum, Trichophyton mentagrophytes* and *Trichophyton rubrum*. After inoculation, the flasks have incubated for 5 weeks at 25 °C in shaken condition at 150 rpm. After incubation, the fungal mycelial growth has excluded through filtration (filter paper Whatman No. 3) and centrifugation at 10000 xg for 10 min. The cell-free supernatant was used for the keratinase assay. (Wawrzkiewicz *et al*., 1987).

Keratinase assay

The activity of keratinase enzyme was determined according to the method described by (Friedrich *et al*., 1999) where 2 ml of the diluted culture filtrate for each fungus were incubated with soluble keratin (20 mg) and 3 ml of phosphate buffer (0.1M, PH 7) at 37 °C for 1h in a shaking water–bath (Gallenkamp BKS-350) at 160 rpm. The enzyme reaction was then stopped by addition of 2 ml of 10 % Trichloroacetic acid (TCA) and the samples were put in the refrigerator at 4 °C for 30 min. The reaction mixture was then centrifuged at 9000 rpm for 20 min in a cooling centrifuge (Hettich Zentrifugen Universal 16/16R). The absorbance was measured at 280 nm using a UV-spectrophotometer. Keratinase activity was determined using a spectrophotometer (Perkin Elmer Hitachi 200). The blank was prepared in the same way but with the exception of addition of TCA before and not after the enzyme reaction. An increase of 0.1 in absorbency was taken to indicate one unit of enzyme activity and one unit of keratinase activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute under optimal experimental conditions.

Effect of AUNPs on Keratinase activity

For testing the effect of AUNPs on keratinase activity of *Aspergillus flavus, Aspergillus niger, Epidermophyton floccosum, Trichophyton mentagrophytes* and *Trichophyton rubrum*, different concentrations of gold nanoparticles (5, 10 and 20 µg/ml) were first incubated with the enzyme of each isolate for 3 hours at room temperature (30 °C). Then incubated with the keratinase induction medium. Inocula treated with AUNPs were compared with inocula treated with fluconazole which used as reference control. The enzyme activity was determined as described previously according to Friedrich *et al*. (1999).

Statistical analysis

The results are expressed as mean±standard error means (SEM). Every experiment was repeated three times. All data were analyzed and compared utilizing one-way ANOVA Duncan test 1995 by using SAS 2004 v.9 and differences with $p < 0.05$ were considered significant (Duncan, 1955; Sas-Nowosielska *et al*., 2004).

RESULTS

In this study a total of 7 species belonging to 4 genera were isolated from 50 patients suspected with the fungal nail infection (onychomycosis).The obtained fungi were *Aspergillus flavus* (8 isolates), *A. niger* (13 isolates), *Candida albicans* (8 isolates), *C. tropicalis* (5 isolates), *Epidermophyton floccosum* (1 isolate), *Trichophyton mentagrophytes* (2 isolates) and *T. rubrum* (1 isolate).

Preparation and characterization of gold nanoparticles

The gold nanoparticles were synthesized with an average particle size of 10 ±2 nm and a concentration of 200 µg/ml. It is clearly that the gold nanoparticles have spherical shape with a well controlled particle size.

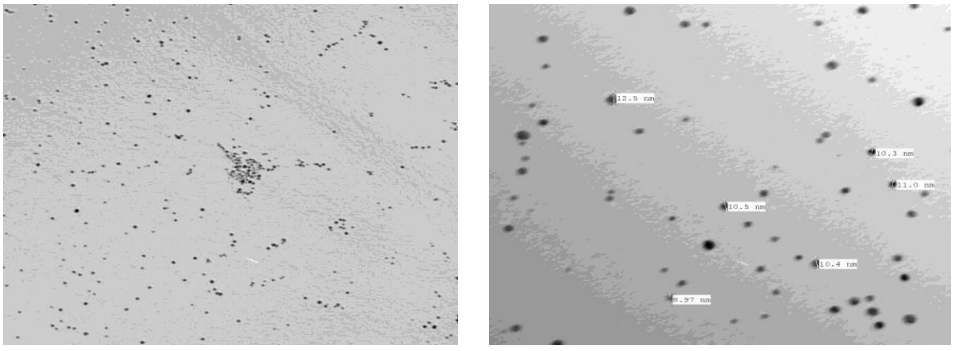

Figure 1: Transmission Electron Microscope (TEM) photos showing the particle size of gold nanoparticles (AUNPs).

Evaluation of antifungal activity of AUNPs

The antifungal activity of AUNPs was tested against *Aspergillus flavus* (8 isolates), *A.niger* (13 isolates), *Candida albicans* (8 isolates), *C.tropicalis* (5 isolates), *Epidermophyton floccosum* (1 isolate), *Trichophyton mentagrophytes* (2 isolates) and *T. rubrum* (1 isolate) by two methods namely agar well diffusion method and micro-dilution method.

Agar well-diffusion method

In the agar well diffusion method, the antifungal effect of AuNPs was expressed on the basis of inhibition zone and the effect differed according to the volume used and according to the tested isolates. The present results revealed the antifungal activities of AuNPs increased with higher volume (100 µl >50 µl >30 µl >10 µl >5 µl) which corresponding to (20 µg, 10 µg, 6 µg, 2 µg and 1 µg respectively) of AuNPs. Using 100µl (= 20 µg) of gold nanoparticles achieved greatest antifungal activity with the best zone of inhibition (19 mm) against *C. albicans* followed by *E. floccosum* (18 mm), C. *tropicalis* (17 mm), *A. flavus* (14 mm) and *A. niger* (12 mm). *Trichophyton* isolates showed resistance to different concentrations of AuNPs. These results were compared to fluconazole as a reference. Statistical analysis showed significant differences ($p<0.001$) between different concentrations of AuNPs compared with fluconazole against the tested fungal isolates (Table 1; Fig.2).

Table 1: Mean inhibition zone of fungal isolates in (mm±SE) against different volumes of gold nanoparticles and fluconazole as a reference

Inhibition zone diameter (mm±SE)								
Fungal isolates	**No. of fungal isolates**	**Fluconazole 10 (µg/ml)**	**AuNPs (µl)**					**P-Value**
			5 (1 µg)	**10 (2 µg)**	**30 (6 µg)**	**50 (10 µg)**	**100 (20 µg)**	
A. flavus	8	11.00 ± 0.57^{cbC}	9.00 ± 0.28^{dD}	11.00 ± 0.76^{cbB}	10.00 ± 0.55^{cC}	12.00 ± 0.57^{bC}	14.00 ± 0.54^{aC}	0.0005***
A. niger	13	9.00 ± 0.57^{cD}	7.00 ± 0.28^{eE}	8.00 ± 0.96^{dC}	8.00 ± 0.55^{dD}	10.00 ± 0.57^{bD}	12.00 ± 0.54^{aD}	0.0004***
C. albicans	8	15.00 ± 0.57^{bcA}	12.00 ± 0.28^{dA}	14.50 ± 1.17^{cA}	15.00 ± 0.55^{bcA}	16.00 ± 0.57^{bA}	19.00 ± 0.54^{aA}	<0.0001***
C. *tropicalis*	5	13.00 ± 0.57^{cB}	11.00 ± 0.28^{eB}	12.50 ± 1.17^{dB}	12.00 ± 0.55^{dB}	14.00 ± 0.57^{bB}	17.00 ± 0.54^{aB}	<0.0001***
E. floccosum	1	13.00 ± 0.57^{cB}	10.00 ± 0.28^{eC}	11.50 ± 1.60^{deB}	12.00 ± 0.55^{dB}	15.00 ± 0.57^{bAB}	18.00 ± 0.54^{aAB}	<0.0001***
T. mentagrophytes	2	10.00 ± 0.57^{aCD}	0^{bF}	0^{bD}	0^{bE}	0^{bE}	0^{bE}	<0.0001***
T. rubrum	1	11.00 ± 0.57^{aC}	0^{bF}	0^{bD}	0^{bE}	0^{bE}	0^{bE}	<0.0001***
P-Value		<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	

[a-b] Values, within a row, with different superscripts are significantly different (***=$p<0.001$)

[A-B] Values, within a column, with different superscripts are significantly different (***=$p<0.001$)

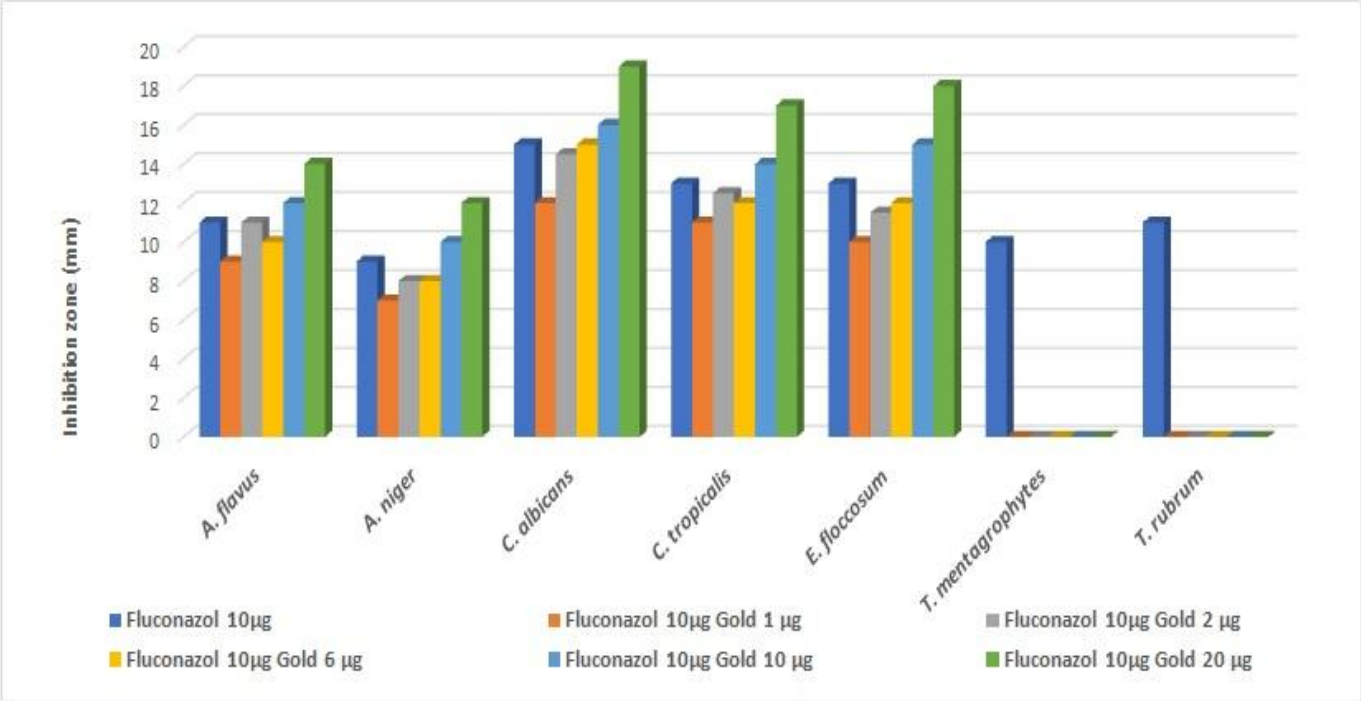

Figure 2: Antifungal effect of AUNPs expressed as inhibition zone (compared to fluconazole)

Micro-dilution method

The current results revealed a significant variation in susceptibility of the tested fungi on application of AuNPs. The results obtained showed that the MIC_{50} and MIC_{90} values of AuNPs ranged from 3.125 to 25.0 µg/ml and from 12.5 to 100 µg/ml respectively, depending on the fungal species. *C. albicans* was the most sensitive to AuNPs with values of 3.125 and 12.5 µg/ml followed by *E. floccosum* 6.25 and 25 µg/ml for MIC_{50} and MIC_{90} respectively. On the other hand, *T. mentagrophytes* and *T. rubrum* were resistant to AuNPs at different concentrations. Statistical analysis showed that there was a significant difference between MIC ranges of gold nanoparticles in comparison with fluconazole against different fungal isolates (Table 2).

Table 2: The mean of MIC_{50} and MIC_{90} (µg/ml) of AuNPs for different fungal isolates in comparison with fluconazole as a reference antifungal drug.

Fungal isolates	No. of fungal isolates	AuNPs (µg/ml)		Fluconazole (µg/ml)		P-Value
		MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	
A. flavus	8	12.50 ± 0.83^{dB}	50.00 ± 1.73^{bB}	16.00 ± 0.57^{cB}	64.00 ± 0.57^{aA}	0.0138**
A. niger	13	25.00 ± 0.83^{dA}	100.00 ± 1.73^{aA}	32.00 ± 0.57^{cA}	64.00 ± 0.57^{bA}	0.0264*
C. albicans	8	3.12 ± 0.83^{cD}	12.50 ± 1.73^{bD}	4.00 ± 0.57^{cD}	16.00 ± 0.57^{aC}	0.0154**
C. tropicalis	5	6.25 ± 0.83^{cC}	50.00 ± 1.73^{aB}	8.00 ± 0.57^{cC}	32.00 ± 0.57^{bB}	0.0187**
E. floccosum	1	6.25 ± 0.83^{dC}	25.00 ± 1.73^{bC}	16.00 ± 0.57^{cB}	64.00 ± 0.57^{aA}	<0.0001***
T. mentagrophytes	2	0^{cE}	0^{cE}	32.00 ± 0.57^{bA}	64.00 ± 1.25^{aA}	<0.0001***
T. rubrum	1	0^{cE}	0^{cE}	16.00 ± 0.57^{bB}	32.00 ± 1.25^{aB}	<0.0001***
P-Value		<0.0001***	<0.0001***	<0.0001***	<0.0001***	

[a-b] Values, within a row, with different superscripts are significantly different (*=p<0.05, **=p<0.01 and ***=p<0.001)

[A-B] Values, within a column, with different superscripts are significantly different (***=p<0.001)

Growth of fungi on keratin medium

The current results revealed significant differences between growth rates of the fungal species tested on keratin medium (P<0.0001). All fungal isolates could grow on keratin medium. Isolates of *A. niger* (13 isolates) were the best for growth on keratin as carbon and nitrogen source with mean radial growth 56.02 mm after 10 days at 25 °C followed by the eight isolates of *A. flavus* recorded mean radial growth of 48.10 mm, *T. rubrum* (26.05mm), *T. mentagrophytes* and *E. floccosum* (21.35mm). Isolates of *C. albicans* (8 isolates) recorded growth diameter with mean value of 10.06 mm while those of *C. tropicalis* (5 isolates) with mean value of 8.41 mm (Table 3; Figure 3a&b).

Table 3: Mean radial growth of dermatophytes, non-dermatophytes and *Candida* species on keratin agar at 25 °C.

Fungal isolates	Mean radial growth (mm)
Aspergillus flavus(n=8)	48.10 ± 2.16^{b}
Aspergillus niger (n=13)	56.02 ± 4.21^{a}
Candida albicans (n=8)	10.06 ± 2.04^{d}
Candida tropicalis (n=5)	8.41 ± 2.18^{d}
Epidermophyton floccosum (n=1)	21.35 ± 1.13^{c}
Trichophyton mentagrophytes (n=2)	21.42 ± 1.22^{c}
Trichophyton rubrum (n=1)	26.05 ± 2.45^{c}

[a-b… etc.] Means, within a column, with different superscripts difference significantly (p<0.0001)

Figures in the table are mean of triplicate.

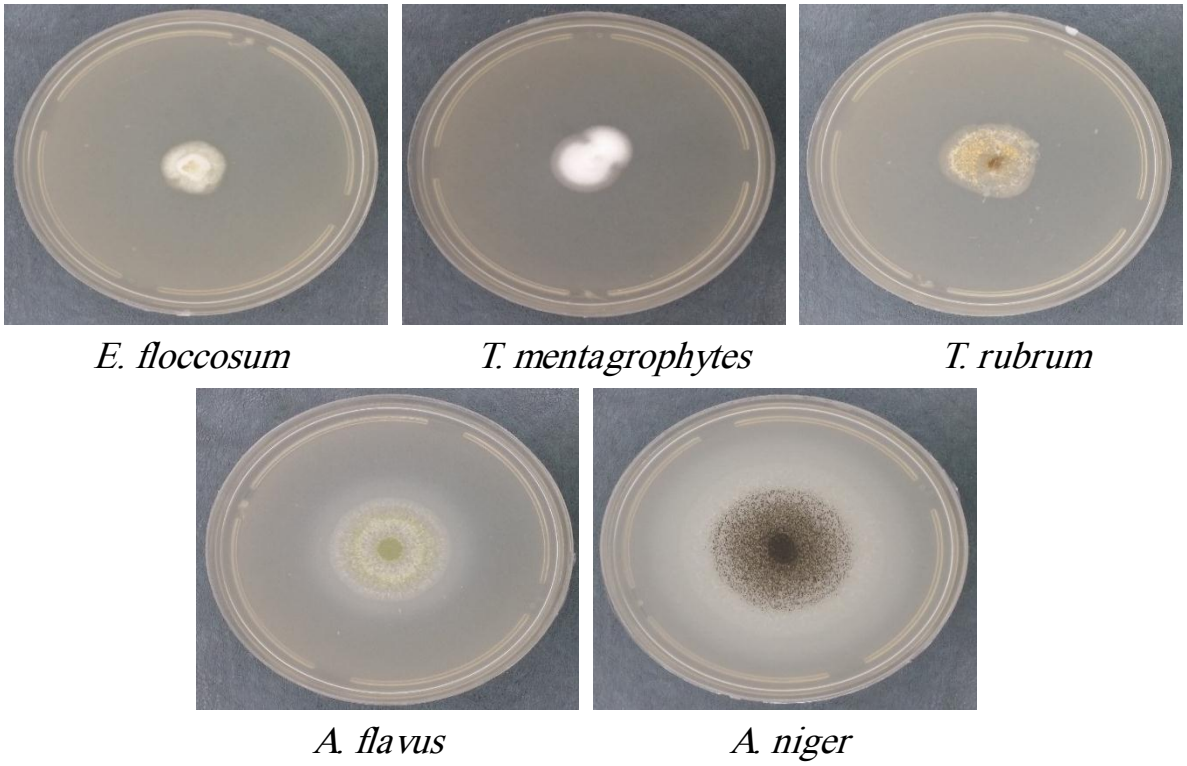

Figure 3: Radial growth of some dermatophytes and non-dermatophytes grown on keratin agar at 25 °C.

Effect of gold nanoparticles on keratinase activity

In the present study, different concentrations of AuNPs were tested on keratinase activity of *A. flavus*, *A. niger*, *E. floccosum*, *T. mentagrophytes* and *T rubrum*, and compared with fluconazole as a reference. The current data showed variation in the keratinase activity of the tested fungal isolates (Table 4). *A. niger* was the most powerful keratinase producer recording an enzyme activity of 40 IU/ml followed by *A. flavus* (30 IU/ml), *Epidermophyton floccosum* (11.5 IU/ml) while *T. rubrum* and *T. mentagrophytes* showed the lowest keratinase activities (8.5 and 7.3 IU/ml respectively). Treatment with different concentrations (5, 10 and 20 µg/ml) of AuNPs induced variable reduction in keratinase activity. Using the 20 µg/ml of AuNPs showed markable reduction in keratinase activity in case of *E. floccosum*, *A. flavus* and *A. niger* of 52.17 %, 40 % and 37.5 % respectively compared with fluconazole. On the other hand, treatment with different concentrations of AuNPs showed no reduction in keratinase enzyme activity of *T. mentagrophytes* and *T. rubrum*. Statistical analysis showed a significant difference between the effect of different concentrations of gold nanoparticles on the keratinase activity compared to fluconazole (Table 4).

Table 4: Effect of different concentrations of gold nanoparticles on the activity of keratinase enzyme produced by some fungal species compared with fluconazole as a reference antifungal drug.

keratinase activity (IU/ml)										
Fungal isolates	**Control**	**AuNPs (µg/ml)**						**Fluconazole (10µg/ml)**		
		5		**10**		**20**				
	Activity	**Activity**	**Reduction (%)**	**Activity**	**Reduction (%)**	**Activity**	**Reduction (%)**	**Activity**	**Reduction (%)**	**P-Value**
A. flavus	30.00±1.15^{aB}	28.00±1.73^{bB}	6.67	25.00±1.73^{dB}	16.67	18.00±1.73^{eB}	40	26.00±1.73^{cB}	13.33	0.0041**
A. niger	40.00±1.15^{aA}	36.00±1.73^{bA}	10	34.00±1.73^{cA}	15	25.00±1.73^{dA}	37.5	37.00±1.73^{bA}	7.5	0.0008***
E. floccosum	11.50±1.15^{aC}	10.50±0.57^{abC}	8.7	9.50±0.57^{bC}	17.39	5.50±0.57^{cC}	52.17	10.00±0.57^{abC}	13.04	0.0016***
T. mentagrophytes	7.00±1.15^{aD}	0^{cD}	0	0^{cD}	0	0^{cD}	0	5.90±0.57^{bD}	19.18	<0.0001***
T. rubrum	8.50±1.15^{aD}	0^{cD}	0	0^{cD}	0	0^{cD}	0	6.10±0.57^{bD}	28.24	<0.0001***
P-Value		<0.0001***		<0.0001***		<0.0001***		<0.0001***		

[a-b] Values, within a row, with different superscripts are significantly different (*=p<0.05, **=p<0.01 and ***=p<0.001)

[A-B] Values, within a column, with different superscripts are significantly different (***=p<0.001)

Discussion

Onychomycosis is a common superficial fungal infection of the nails leading to discoloration, nail plate thickening, and onycholysis. Mycotic nail disease is the most common nail pathology worldwide, reaching all cultures and ethnicities. Onychomycosis is increasing, accounting for up to 90 % of toenail and at least 50 % of fingernail infections (Ghannoum *et al*., 2000). In the present study, 50 patients were screened for incidence of onychomycosis and included in this study after obtaining approval of the Dermatology Research Ethical Committee of the Faculty of Medicine, Cairo University, study was performed during June 2015 to May 2016.

In this study, it was possible to isolate and identify 7 species belonging to 4 genera from patients suffering from onychomycosis disease. Dermatophytes isolated from 4 cases, of which *T. mentagrophytes* was the common recorded in 2 cases, while *E. floccosum* and *T. rubrum* were recorded only in one case Non-dermatophytes; *A.*

flavus (8 cases) and *A. niger* (13 cases). *Candida* was represented by *C. albicans* and *C. tropicalis*; the first was isolated from 8 cases and the later from 5 cases.

In disagreement with the current results Bedaiwy *et al*. (2017) recorded in their study performed in Benha University Outpatient Clinics that yeasts followed by dermatophytes and non-dermatophytes were the most frequently isolated fungi comprised 56.0 %, 36.0 % and 8.0 % of total fungi respectively. Wajid *et al*. (2016) in their study found that *Candida* species was isolated from 52.0 % of cases. On the other hand, Khader *et al*. (2015) in their study reported that dermatophytes were the most prevalent species (72.7 %) followed by non-dermatophytic molds. These variations in fungal incidence varied according to the season of sampling and isolation.

Nowadays the field of nanotechnology becomes one of the most topics of interest because nanostructures exhibit chemical and physical properties which are distinctly different from the bulk solid (Weller, 1993) and application and the use of (NPs) in different applications is emerging rapidly. One of the most characteristics of gold nanoparticles (AuNPs) is that they have large surface area and dispersion due to their very small size and proper shapes. These properties make gold nanoparticles being topic of interest and applications in many fields especially in the field of medicines (Huang and El-Sayed, 2010).

In the current study, the antifungal effect of gold nanoparticles against the tested fungal isolates was evaluated by using agar well-diffusion method and micro-dilution method. In the first method, the antifungal effect of AuNPs against the tested fungal isolates increased with increasing volume of AuNPs compared with fluconazole as a reference. This effect was expressed as a diameter of inhibition zone on agar plates with different volumes of gold nanoparticles (5, 10, 30, 50 and 100 µl). The best antifungal activity of gold nanoparticles was observed by using 100 µl (= 20 µg of AuNps) with the greatest zone of inhibition reaching 19 mm against *C. albicans* and 18 mm against *E. floccosum*. In this respect, Zawrah *et al*. (2011) used spherical gold nanoparticles coated with CTAB and they found that the best antifungal activity (expressed as inhibition zones) was obtained against *A. niger* (14 mm), *C. albicans* (13 mm) and *A. flavus* (12 mm). Another method of green synthesis of gold nanoparticles from the aqueous extract of *Abelmoschus esculentus* was used by Jayaseelan *et al*. (2013) with the best zone of inhibition against being recorded against *C. albicans* (18 mm), *A. flavus* (16 mm) and *A. niger* (15 mm) when using 50 µl of gold nanoparticles and they reported that the gold nanoparticles were effective against all the fungal species tested.

Folorunso *et al*. (2019) found that AuNPs synthesized from leaf extracts of *Annona muricata* showed the broad and the highest percentage inhibition (66 %) against *Penicillium camemberti* at a concentration of 4 mg/ml. On the other hand, the lowest percentage of inhibition zone (30 %) was recorded in case of *A. flavus* at a concentration of 2 mg/ml. So, it was noted in their study that the antimicrobial effect of AuNPs increases with increasing concentration. Ahmad *et al*. (2013) reported that the gold nanoparticles exhibited excellent size dependant antifungal effect against *Candida* isolates and they found that sensitivity index, which is calculated as diameter of inhibition zone /drug concentration, for *C. albicans* was maximum (4.2) when treated

with 7 nm gold nanoparticles and was minimum (1.1) for *C. glabrata* when treated with 15 nm gold nanoparticles.

Jebali *et al*. (2014) studied the effect of different shapes of gold nanoparticles and evaluated that gold nanocubes had the higher antifungal effect against *Candida* species than nanospheres and nanowires with best inhibition zone obtained on applying gold nanocubes recording 17.1, 17.0 and 16.0 mm for *C. albicans, C. glabrata* and *C. tropicalis* respectively. In the present study, it was shown that *T. rubrum* and *T. mentagrophytes* were resistant to AuNPs at different levels and this was agreed with a study performed by Rónavári et al. (2018) who found that *T. mentagrophytes, T. tonsurans* and *Microsporum gypseum* were resistant to AuNPs even up to 300 µg/ml with no inhibition on fungal growth.

Regarding micro-dilution method, AuNPs showed variable MIC ranges against the tested fungal isolates and the results obtained showed that the MIC_{50} and MIC_{90} values of AuNPs ranged from 3.125 to 25.0 µg/ml and 12.5 to100 µg/ml respectively depending on the fungal species. In this respect, Ahmad *et al*. (2013) reported that the MIC values of 7 nm gold nanoparticles against *C. albicans, C. tropicalis* and *C. glabrata* were found to be 4.0, 4.0 and 8.0 µg/ml respectively. Jebali *et al*. (2014) found that MIC_{50} for *C. albicans, C. glabrata* and *C. tropicalis* were 3, 10 and 11 µg/ml respectively, while MIC_{90} for the same isolates were 15, 47, 48 µg/ml respectively on application of spherical gold nanoparticles.

Also, Zawrah *et al*. (2011) found that the MIC for *C. albicans, A. niger* and *A. flavus* were 6.25, 6.25 and 12.5 respectively. It was found that metal nanoparticles have inhibitory effect on bacteria and fungi due to destroying cell membrane integrity (Chwalibog *et al*., 2010; Ravishankar Rai and Jamuna Bai, 2011). The mechanism of gold nanoparticles to inhibit bacterial and fungal growth was described and stated as gold nanoparticles stimulate biofilm production and aggregate within this biofilm. They attach and bind closely to the surface of microorganisms causing visible damage to the cells and destruction. Gold nanoparticles have well-developed surface chemistry, chemical stability and appropriate smaller size, which make them easier to interact with the microorganisms (Grace and Pandian, 2007). As a result, structural changes and damage especially disturbing the vital functions of the cells such as permeability, making pits and gaps making a depression of the activity of respiratory chain enzymes, interact with the major building elements of the outer membrane and might cause structural changes, degradation and finally leading to cell death.

In the current study, a group of dermatophytes (*E. floccosum, T. mentagrophytes* and *T. rubrum*) and non-dermatophytes (*A. flavus, A. niger, C. albicans,* and *C. tropicalis*) were screened for their ability to grow on keratin medium and degrade keratin. *A. niger* showed the highest ability of keratin degradation and recorded colony diameter of 56.02 mm after 10 days at 25 °C followed by *A. flavus* (48.10 mm), *T. rubrum* (26.05 mm), *T. mentagrophytes* (21.4) *E. floccosum* (21.35) and *Candida* isolates recorded 10.06 and 8.41 mm for *C. albicans* and *C.tropicalis* respectively. Keratinase activity of keratinophilic fungi is considered one of the most important concepts associated with their ability to infect a wide range of living organisms. Keratinolytic fungi have an important ecological interest not only in pathogenesis but

also in keratin degradation. In harmony with the current findings, Sharma *et al*. (2011) studied the keratinolytic activities of some dermatophytes and yeast species and they reported that *T. mentagrophytes* could degrade the keratin and showed a colony diameter of 49.0 mm on the keratin medium while *T. rubrum* gave 45.0 mm and *C. albicans* attained a colony diameter 40.0 mm. Also, Mazotto *et al*. (2013) found that four *Aspergillus niger* strains could grow on keratin medium resulting in the degradation of keratin and formation of colony diameter ranged between 5.0 and 35.0 mm. So, the degradative enzymes (keratinase enzyme) produced by keratinolytic fungi are able to break down complex keratinous substrates in nature and this leading to biodegradation of keratin in polluted habitats.

In the current study, soluble keratin was prepared and used for keratinase production and assay for the fungal isolates which had the greatest colony on keratin medium, these namely *A. flavus, A. niger, E. floccosum, T. mentagrophytes* and *T. rubrum*. The present results showed variation in the enzyme activity of different fungal isolates. *A. niger* was the most powerful keratinase producer with keratinase activity of 40 IU/ml followed by *A. flavus* (30 IU/ml) and *Epidermophyton floccosum* (11.5 IU/ml), while *T. rubrum* and *T. mentagrophytes* showed lower keratinase activities of 8.5 and 7.3 IU/ml respectively. In this respect, Mazotto et al. (2013) found that *A. niger* 3T5B8 had the highest keratinase activity (172.7 IU/ml) after seven days at pH 5 in solid-state fermentation while the lowest activity (21.3 U/ml) was recorded by *A. niger* 9D40 after four days in submerged fermentation. Also, Ouf et al. (2015) found that *M. gypseum* was the most powerful keratinase producer (38.0 IU mg-1 dry weight) followed by *M. canis* and *E. floccosum* (18.1 and 16.5 IU mg-1 dry weight, respectively). *T. mentagrophytes* and *T. rubrum* showed lower keratinase activities of 8.5 and 7.3 IU mg-1 dry weight respectively. Another study of Kim (2003) who found that higher (12.9 KU/ml) and lower (10.4 KU/ml) keratinase activity was recorded by *A. flavus* and *A. fumigatus* respectively .

In the present study we evaluate the effect of gold nanoparticles on the activity of keratinase enzyme produced by *A. flavus, A. niger, E. floccosum, T. mentagrophytes* and *T. rubrum*, and it was found that the treatment with different concentrations (5, 10 and 20 µg/ml) of AuNPs induced variable reduction in keratinase activity. Using 20 µg/ml of AuNPs showed markable reduction in the activity of keratinase of *E. floccosum, A. flavus* and *A. niger* recording 52.17 %, 40 % and 37.5 % respectively if compared with fluconazole as a reference. It was also notice in the current study that the treatment with different concentrations of AuNPs showed no reduction in the enzyme activity of keratinase for *T. mentagrophytes* and *T. rubrum* as compared to control.

Conclusion

Onychomycosis is a common fungal nail infection affecting both fingernails and toenails which usually caused by dermatophytes, yeasts and molds. The application of gold nanoparticles in many fields becomes the topic of interest in recent days. On the basis of our results of this study, we can conclude that AUNPs have inhibitory effect on the fungal growth and can reduce the keratinase enzyme activity of the tested fungi. Fluconazole is a common antifungal drug used to treat onychomycosis but AUNPs are more effective than fluconazole especially at higher concentrations.

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تأثير جزيئات الذهب متناهية الصغر علي الفطريات المسببة للعدوي الفطرية للأظافر

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العدوى الفطرية للاظافر هى عدوى فطرية منتشرة على نطاق واسع والتي يمكن أن تسببها الفطريات الجلدية أو الخمائر أو الفطريات غير الجلدية. فى الاونة الاخيرة اصبح مجال النانو هو الاكثر شيوعا فى كل المجالات .فى دراستنا تم تطبيق ودراسة تاثير جزيئات الذهب متناهية الصغر على بعض الفطريات المسببة للعدوى .هذه الفطريات تم عزلها من ٥٠مريض يعانون من فطرالاظافر .ووجدنا ان جزيئات الذهب لها تاثير فعال على نمو الفطريات وان اقل تركيز مثبط ل ٥٠% و ٩٠%من نمو الفطريات يتراوح مابين٣.١٢٥ الي ٢٥ و ١٢.٥ الي ١٠٠ ميكروجرام/مللى على التوالى .هذه الفطريات المسببة للعدوى لها القدرة على تحليل الكيراتين لافرازها لانزيم الكيراتينيز وقد وجدنا ان جزيئات الذهب متناهية الصغر لها القدرة على تثبيط نشاط الانزيم وقد قارنا النتائج بالمضاد الفطرى الفلوكانازول واثبتنا ان جزيئات الذهب متناهية الصغر لها تاثير فعال ومثبط للفطريات اكثرمن الفلوكانازول وخاصة عند التركيزات العالية.