

A COMET ASSAY FOR CANDIDA GLABRATA INFECTION AND TREATMENT BY PENICILLIUM CHRYSOGENUM - DERIVED AgNPs

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ABSTRACT

The colonies of *C. glabrata* were described, which morphology of isolated on Sabouraud dextrose agar with chloramphenicol. Biosynthesis of nanoparticles is of great importance since they are highly involved in various medical and biological applications. The current study aims to produce *Penicillium chrysogenum* - derived AgNPs to be used to treat *Candida glabrata*. Nanoparticles were formed, the results of which was observed through UV-visible spectrophotometry. Therefore, the surface morphology and particles size of AgNPs were assessed using the scanning electron microscope (SEM). The atomic force microscopy (AFM) confirmed that the average diameter of AgNPs was 18 nm which is appropriate to be used to deliver *Penicillium chrysogenum* to the *Candida* infected cells. The antifungal activity of AgNPs against *C. glabrata* was investigated in the serum of 15 mice. The mice were divided into four groups was examined after 21 days. A comet assay described procedure that sensitive measurement and permits reproducible of DNA repair and DNA damage using *C. glabrata* infection and treatment by *Penicillium chrysogenum* - derived AgNPs. This combination of assay with affected *C. glabrata* in DNA repaired and the extensive range of obtainable fungal molecular biology apparatuses can donate to light important devices of genome.

Key word: Nanoparticles, comet assay

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تحليل المذنب لعدوى *Candida glabrata* وعلاجها بواسطة AgNPs المشتق من *Penicillium chrysogenum*

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مدرس

قسم علوم الحياة، كلية العلوم للبنات، جامعة بغداد، بغداد، العراق.

المستخلص

تم وصف مستعمرات نبات *C. glabrata* والتي تم عزلها على وسط Sabouraud dextrose مع الكلورامفينيكول. يعتبر التخليق الحيوي للجسيمات النانوية ذا أهمية كبيرة لأنها تشارك بشكل كبير في مختلف التطبيقات الطبية والبيولوجية. تهدف الدراسة الحالية إلى إنتاج AgNPs المشتق من *Penicillium chrysogenum* لاستخدامها في علاج الإصابة بـ *Candida glabrata*. تم تشكيل الجسيمات النانوية والتي لوحظ تكوينها بواسطة جهاز القياس الطيفي المرئي للأشعة فوق البنفسجية. تم تقييم التشكل السطحي وحجم الجسيمات لـ AgNPs باستخدام المجهر الإلكتروني الماسح (SEM). كذلك تم تأكيد الفحص المجهرية للقوة الذرية (AFM) حيث وجد أن متوسط قطر AgNPs كان 18 نانومتر وهو مناسب لاستخدامه لإيصال *Penicillium chrysogenum* إلى الخلايا المصابة بـ *Candida*. تم فحص النشاط المضاد للفطريات لـ AgNPs ضد *C. glabrata* في مصل 15 فأر. حيث تم تقسيم الفئران إلى أربع مجموعات تم فحصها بعد 21 يوماً. تم وصف اختبار المذنب الذي يسمح بقياس الحساسية لتلف الحمض النووي وإصلاح الحمض النووي جراء عدوى *C. glabrata* والعلاج بواسطة AgNPs المشتق من *Penicillium chrysogenum*. يمكن أن يتم الجمع بين هذا الاختبار مع *C. glabrata* والذي يتأثر بصيانة الحمض النووي ومجموعة واسعة من أدوات البيولوجيا الجزيئية للخميرة التي يمكن الحصول عليها لإضاءة الآليات المهمة للجينوم.

الكلمات المفتاحية: خمائر الكانديدا، الجزيئات النانوية، فطر البنسيليوم فحص المذنب.

INTRUDUCTION

Candida glabrata is an opportunistic that causes human illness in a proportion about to 29% of total causes *Candida* bloodstream infections (18). It infection occupies the third or fourth position, in addition to its widespread prevalence, causing systemic diseases in Asia (8). *C. glabrata* are often found in aging individuals, solid organ transplant receivers and diabetic patients (15). It's also a causal agent of vaginal and urinary tract infections (1). Even though anti-mycosis treatment be useless because of the high resistance of *C. glabrata* to numerous treatments (21), systemic infections of *C. glabrata* frequently result in high mortality rate (17). *Candida* is difficult to diagnose because the symptoms and clinical signs are commonly non-specific (12). A more-sensitive technique for the earliest possible analysis of *Candida* bloodstream infections and then starting antifungal therapy is essential for more prognoses (16). Filamentous fungi have a great advantage to using with nanoparticle synthesis. For example, fungi need simple nutritional requirements, easy to get, have great wall-binding size, and high absorption capabilities (24). The *Penicillium chrysogenum* is characterized by being fast growing on the medium and has the importance of being an antimicrobial, so it was used for the biosynthesis of silver nanoparticles (23). Mice are widely-used in laboratory experiments as animal models, where we can diagnose different types of clinical *Candida* (2,26). The comet assay (single-cell gel electrophoresis) is a useful, simple, and sensitive measurement assay that is previously widely applied to study many characteristics of DNA damage (9). The DNA damage stimulated by *Candida glabrata* infection and repair degree of DNA damage stimulated by biosynthesis of silver nanoparticles have been assessed through various organs of the infected mouse by the comet assay (12,19). In this study, with the help of previous procedures to fit for *C. glabrata* cells and optimizing suitable for the assay, the basic comet assay was used to study the infection-treatment relationship for *C. glabrata* cell DNA damage after treated by biosynthesis of silver nanoparticles with *Penicillium chrysogenum*. The mouse model

was serious discovered in studies aiming to explain mechanisms of pathogenicity carried out by *Candida glabrata* and the use of silver nanoparticles biosynthesis with *Penicillium chrysogenum* as a treatment after infecting mice with this yeast detected by comet assay.

MATERIALS AND METHODS

Medium and growth conditions: The blood samples were collected from patients with candidaemia from Al-yarmook hospital in Baghdad. *Candida glabrata* was cultured on Sabouraud dextrose agar (SDA) (Himedia, India) with chloramphenicol for (24 hours at 37 °C). *Candida* Cell were then inoculated in appropriate media (Sabouraud dextrose broth SDB ,Himedia, India) and incubated for 24 h at 37 °C (10). The subculture was pelleted and washed twice with 0.01 M phosphate buffered saline (PBS) have pH 7.2. The final pellet was re-suspended in PBS; the concentration of blasto-pores was totaled with a spectrophotometer to give $2 * 10^4$ blasto-spores/ml for the in vitro assays in mice.

Synthesis of *Penicillium chrysogenum* - derived AgNPs: *Penicillium chrysogenum* - derived AgNPs were synthesized by taking 0.2 ml solution of AgNO₃ in a 10 ml test tube, followed by deionized water then 0.2 ml *Penicillium chrysogenum* were grown in appropriate liquid medium (KH₂PO₄ 7.0; MgSO₄. 7H₂O 0.1; (NH₄)₂SO₄ 1.0; and glucose 10.0 g\L) and then 0.1 ml NaOH, final volume was 5 ml.

UV-Vis measurement: The UV-Vis measurement was done using a UV-Vis (V670 JASCO, Japan).

Atomic force microscope: The dimension of AgNPs was measuring using atomic force microscope AFM (AA- 300, Shimadzu-Japan).

Scanning electron microscope: The morphology of AgNPs was performed using scanning electron microscope SEM (TESCAN, Czech Republic). Preparation of *Penicillium chrysogenum* - derived AgNPs was re-formed with 5 ml sterile water and shaken dynamically for 1 min for injection. This resulted in a 10-mg/ml solution of *Penicillium chrysogenum* - derived AgNPs which was diluted for i.v. injection

Experimental Animals

This experiment was examining the possibility of using the application of *Penicillium*

chrysogenum - derived AgNPs as antifungal activity against *C.glabrata* in mice. Twelve male there are in good health mice (6 weeks old) their weigh ranged from 20-25 gm were the ones used in this study. Special mice were saved for the experiment in stainless steel cages in temperature rate ($22\pm 1C^{\circ}$); moisture was at $55\pm 10\%$ and providing nutrition with water. The mice animals in cages were divided into four groups for testes, three mice in group according to administration:

1. Control group which administrated 1 ml physiological saline solution.
2. *C. glabrata* group (none treated), animal groups were infected with $2 * 10^4$ cells/ml cells of *C. glabrata*.
3. AgNPs group, administration with *Penicillium chrysogenum* - derived AgNPs only (70 μ l).
4. Treated group, *C. glabrata* infected in mice during one week after that treated by *Penicillium chrysogenum* - derived AgNPs (5). The animal groups were administered daily for 21 day. The fungal assessment was carried out every 1day for 21 day after managements to avoid the can reduce of *Candida* load in the tissue. After 21 days of treatment, mice were killed by anesthetic (Ketamine-Xylazine, mouse).

Alkaline comet assay

The electrophoresis situations used will regulate the sensitivity outcomes of the comet assay. It has two areas: (Neutral Comet Assay) will identify double-stranded DNA breaks, whereas (Alkaline Comet Assay) will discover single and double-stranded DNA breaks, and the mainstream of basic places in addition to alkali labile DNA adducts (*e.g.* phosphoglycols, phosphotriesters). Lysis Solution was primed and cooled at $4^{\circ}C$ for 20 minutes before usage. LM Agarose was melted in a glass of boiling water for 5 minutes. Bottle was sited in $37^{\circ}C$ water bath for 20 min. Cells were joint at 1×10^5 /mL with molten LM Agarose (at $37^{\circ}C$) at a relation of 1:10 (v/v) and directly pipetted 50 μ l onto comet slide. Slides flat were located at $4^{\circ}C$ for 10 minutes. A 0.5 mm clear ring seems at edge comet slide

zone. Increasing forming time to 30 minutes advances adherence of samples in high humidity conditions. Slides were absorbed in $4^{\circ}C$ for 30-60 minutes. Samples were dehydrated at $37^{\circ}C$ for 10-15 minutes. Drying transports all the cells in a particular plane to enable observation. 100 μ l of diluted (SYBR Green) was retained onto each circle of dried agarose and stained 30 minutes (room temperature) in the dark. Slide was tapped to remove additional SYBR solution and rinsed in water. Slides were completely dried at $37^{\circ}C$ for watched by fluorescence microscopy, (SYBR Green's maximum excitation/emission is 496 nm/522 nm.), fluorescein filter is adequate) (6). The technique of quantification by using (image examination software comet score), it will analyze different factors for each comet. Three limits were estimated to designate DNA migration, tail length described as (distance from the center of the head to the end of the tail), it is calculated as follows:

Number of tails produced

DNA /total DNA

tail DNA%=100X.

- Tail DNA Intensity/Cell DNA Intensity (13).

RESULTS AND DISSCUTION

C. glabrata are recognized with procedures shining, smooth and milky stained colonies which are medium fuzzy from other *Candida* spp, the exception of their comparative size, which is lesser, the only *C. glabrata* that does not procedure pseudo-hypha at temperatures $37^{\circ}C$. The presence of *Penicillium chrysogenum* - derived AgNPs has been confirmed by UV-visible Bio-spectrophotometry. For the strong absorbance measurements of this experiment will filtrate for 2 hours for scan wavelength in the range of (200 to 600) nm. The outcomes display that the strong absorbance value peak occurred between (220-430) nm when detected through using UV-visible Bio-spectrophotometry. Characterization of *Penicillium chrysogenum* - derived AgNPs was checked using AFM. It was investigated that, nanoparticles of size 18 nm were shaped in (Figure 1).

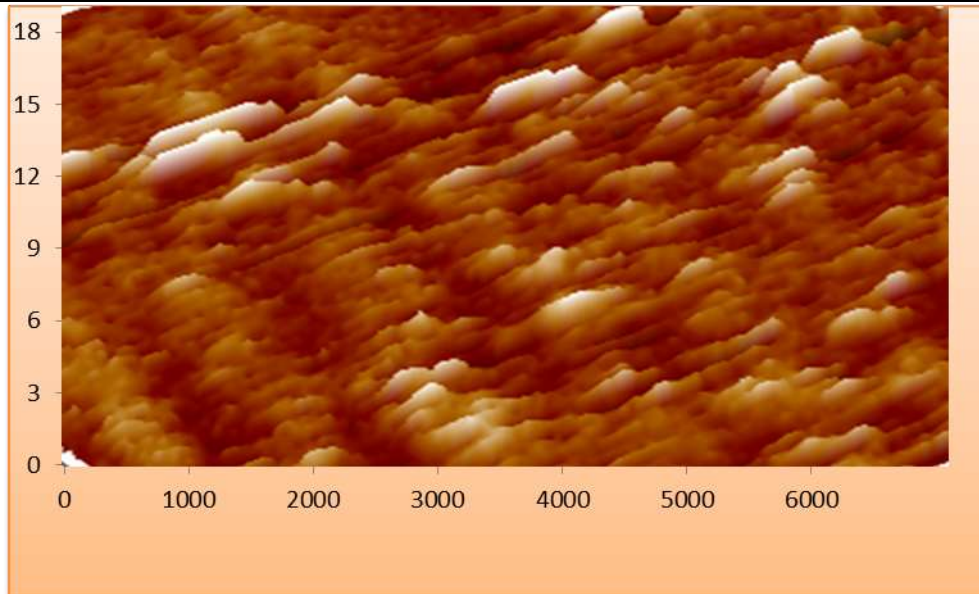


Figure 1. AFM image of silver nanoparticle having a size of 18nm

Characterization of *Penicillium chrysogenum* - derived AgNPs was carried out using SEM in (Figure 2).

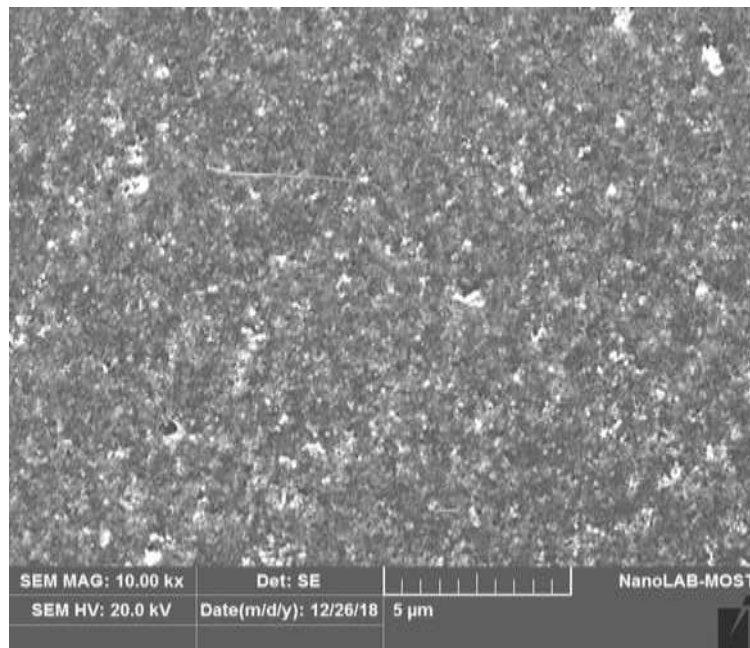


Figure 2. SEM image of silver nanoparticle

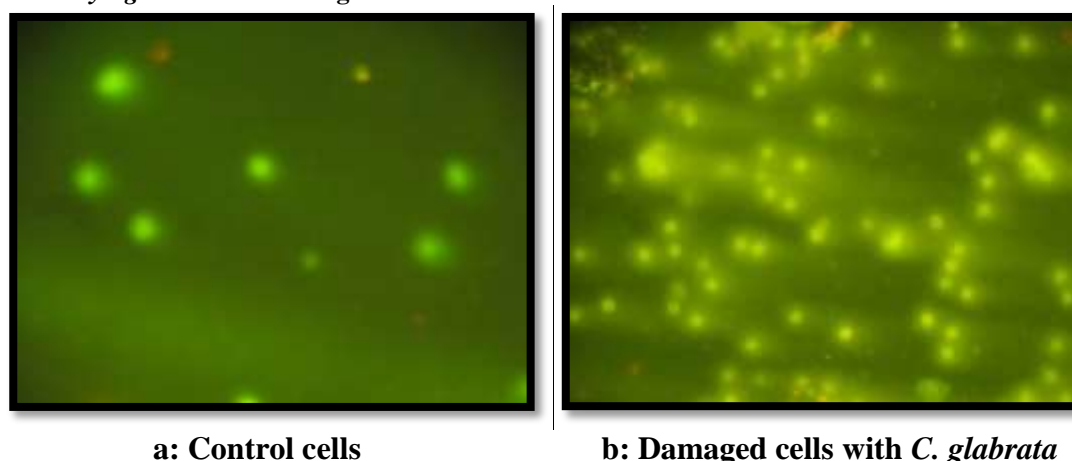
The serum of 12 mice divided into 4 groups was examined. All the totals of containing 3 animals blood were withdrawn from them to obtain serum for comet analysis after the 21 day. Single cell gel (SCG) documents the detection of DNA alternate of different types, similar double and single strand breaks, imperfect repair sites, alkali-labile sites, cross links and repair in different cells (22). Comet assay technique including the use of a number of sensitive reagents that catch the DNA defects in addition describe amount of DNA by measuring the relationship between the genetic

contents of the materials and the resulting tail. A sample of 12 mice (*C. glabrata* group (none treated), AgNPs group, treated group and control, were used to this study. Results in Table 1 and (Figure 3. a, b, c, d) shows that the tail length, tail and tail mean instant was meaningfully *C. glabrata* group (none treated), percent of DNA in AgNPs group and treated group respectively as compared with controls. Three examples of scoring categories for comet assay (a: Normal, b: DNA Damage; c: Control positive group; d: treated group) in mice cells.

Table 1. Percentage frequency of DNA damages in mice and control using comet assay

Control group	NO.	%	Low	%	Medium	%	High	%	Sum.	Sum.
1	74	41.11	81	45.00	14	7.78	11	6.11	180	100
2	87	48.88	69	38.76	12	6.74	10	5.62	178	100
3	63	45.32	57	41.01	9	6.47	10	7.19	139	100
<i>C. glabrata</i>	NO.	%	Low	%	Medium	%	High	%	Sum.	Sum.
1	46	25.27	51	28.02	48	26.37	37	20.33	182	100
2	36	29.03	28	22.58	27	21.77	33	26.61	124	100
3	51	29.14	45	25.71	38	21.71	41	23.43	175	100
<i>Penicillium-nano</i>	NO.	%	Low	%	Medium	%	High	%	Sum.	Sum.
1	54	46.96	48	41.74	7	6.09	6	5.22	115	100
2	64	46.72	55	40.15	9	6.57	9	6.57	137	100
3	77	49.04	63	40.13	8	5.10	9	5.73	157	100
<i>Candida</i> treated with <i>Penicillium -nano</i>	NO.	%	Low	%	Medium	%	High	%	Sum.	Sum.
1	55	36.67	58	38.67	23	15.33	14	9.33	150	100
2	81	37.85	77	35.98	35	16.36	21	9.81	214	100
3	65	40.12	53	32.72	28	17.28	16	9.88	162	100

N0.: number a: Control cells b: Damaged cells with *C. glabrata* c: AgNPs only d: Treated with *Penicillium chrysogenum* - derived AgNPs



a: Control cells

b: Damaged cells with *C. glabrata*

Figure 3. The comet assay for (a: Untreated cells or control cells, b: Damaged cells with *C. glabrata*, c: Control positive with AgNPs only, d: cells treated) with *Penicillium chrysogenum* - derived AgNPs

Due to the medical importance of *C. glabrata* blood stream infections in population, the effectiveness of treatments against yeast infections is progressively applied in mice models (7). The results of clinical data showed that immune-compromised patients are more susceptible to infection with *C. glabrata* infections and with patient effected candidiasis (3). In mice, certain features of the host resistance are clearly complicated in *C. glabrata* survival in blood stream infections (20). *P. chrysogenum* was availed for the biological synthesis of nanoparticles extracellular. Green synthesis of silver nanoparticles method is considered useful

chemical approaches as it is economic and environmentally friendly (14). Such applications as (bactericidal, medical and electronic applications create), therefore, this method can stimulating for high production of synthesis from other mineral materials (Nano-materials). *P. chrysogenum* was considered one of the best for production AgNPs at average a pH 6, at aoptimum concentration of 0.1 ml NaOH with a substrate concentration of 0.2 ml AgNO3. The size of the partials was observed to be 18 nm by AFM which is a good result that facilitates the synthesis process with treatment ; about the morphological studies are accomplished by SEM which shows the

appropriate from the right employment. Antimicrobial activities of AgNPs produced by *p. chrysogenum* were studied against *C. glabrata*. By identifying the figure 2., we can concluded that the most of the silver nanoparticles have the shape to be triangle, others are pyramid shaped and some are spherical in nature. Where in figure 3., *C. glabrata* group showed highest fold increase with non- treated compared with treated group with *Penicillium chrysogenum* - derived AgNPs (4). Comet assay is an assistance analysis in measuring DNA defects and is a significant requirement in men opting for *C. glabrata* blood stream infection. The true rate of DNA breaks were assessed by using comet assay. These results might be as many reasons: masking, overlapping and tangling of fragments that transfer, imperfect chromatin strengthening. The cell walls of *C. glabrata* surrounding the protoplasm and this feature is acceptable the comet assay makes to be completed on *C. glabrata* and optimized (25).

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