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Treatment isolated fungi from laboratory tools in some Baghdad hospitals by using biosynthesized nanoparticles

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Abstract

The study aims to biosynthesized of sliver nanoparticle from aqueous extract of olive leave and evaluate the effectiveness of the synthesis AgNPs against isolated fungi. The study mediating fifty samples were taken from various tools in laboratory from five hospitals in Baghdad. Four species of fungi were identified depending on the morphological and microscopic characteristics. The most common isolated fungi based on their frequency ratio were as follows *Aspergillus niger* 87.5%, *Aspergillus flavus* 62.5%, *Aspergillus fumigatus* 53.5% and *Aspergillus nidulans* 37.7%.The Biosynthesis of silver nanoparticle developed a rapid, eco-friendly and convenient green method for the stable silver nanoparticles (AgNPs) were synthesised with an average diameter of 30 ± 60 nm and like spherical in shape, using the aqueous solution of the Olive tree (*Olea europaea*) leaves extract.The reaction is carried out at 10^{-3}M of silver nitrate. The AgNPs synthesized were confirmed by their change of color to (dark brown-grey). The characterization was studied using UV-Visible spectroscopy, Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). Inhibition effect of AgNPs against fungi has been studied using well diffusion method by studying the effect of different concentration (100, 75, 50 and 25). The results revealed that the AgNPs have considerable antifungal activity comparison with alcohol. The obtained results indicate that the highest level of inhibition zone was detected at the concentration of 100 µg/ml of AgNPs, where the inhibition zones are (23.33 ± 4.41) for *A. flavus* and the lowest level of inhibition zone was detected at the concentration 25 µg/ml of AgNPs ,where the inhibition zones are (6.00 ± 1.15) for *A.nidalus*. While using alcohol the highest level of inhibition zone was detected at the concentration of 100 µg/ml of Alcohol, where the inhibition zones are (12.33 ± 1.45) for *A.nidalus*, and the lowest level of inhibition zone was detected at the concentration 25 µg/ml of Alcohol ,where the inhibition zones are (4.67± 0.33) for *A.flavus.*

Keywords: sliver nanoparticles, olive leaf extract, *Aspergillus* spp, Inhibition zone.

معالجة الفطريات المعزولة من الادوات المختبرية من بعض مستشفيات بغداد باستعمال الجزيئات النانوية المصنعة حيويا

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الخلاصة

هدفت الدراسة إلى التصنيع الحيوي لجزيئات الفضة النانوية من المستخلص المائي لأوراق الزيتون وتقييم فعالية AgNPs المصنعة ضد الفطريات المعزولة. شملت الدراسة 50 عينة تم اخذها باستعمال مسحات معقمة من ادوات مختلفة في المختبر من 5 مستشفيات في بغداد. تم تشخيص جميع العزلات الفطرية اعتمادا

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على المظهر الخارجي والفحوصات المجهرية. كانت الفطريات المعزولة الاكثر شيوعآ اعتماداً على نسبة ترددها كما يلي: *Aspergillus niger* 87.5% *A.flavus* 62.5% *A.fumigatus* , 53.5% و *A.nidulans*37.7% .تم تحضير المستخلص المائي لأوراق الزيتون وتخليق الجزيئات النانوية من اوراق الزيتون بطريقة صديقة للبيئة بتفاعلها مع نترات الفضة بتركيز 10^{-3} مولاري وتم الاستدلال على جزيئات الفضة النانوية بالتغير اللوني الى اللون البني الغامق الرمادي وتم دراسة الصفات النانوية باستعمال التحليل الطيفي المرئي بالاشعة فوق البنفسجية والفحص بالمجهر الالكتروني (SEM) ومجهر القوة الذرية (AFM)وكانت جزيئات الفضة النانوية ذات متوسط قطر 60 ± 30 نانوميتر وشكل كروي وتم دراسة التاثير التثبيطي لجزيئات الفضة النانوية ضد الفطريات المعزولة باستعمال تراكيز مختلفة(100,75,50,25) وكان له تاثير مضاد للفطريات اعلى من الكحول .تشير النتائج إلى أن أعلى مستوى من منطقة التثبيط تم اكتشافه عند تركيز100 µg / ml من AgNPs ، حيث كانت مناطق التثبيط (4.41 ±23.33) لـ *A. flavus* وأقل مستوى لمنطقة التثبيط تم اكتشافه عند التركيز 25 µg / ml من AgNPs ، حيث تكون مناطق التثبيط (1.15 ± 6.00) لـ . *A.nidalus* بينما أثناء استخدام الكحول تم اكتشاف أعلى مستوى من منطقة التثبيط عند التركيز 100µg / ml ، حيث تكون مناطق التثبيط (1.45 ±12.33) ل *A.nidalus* ، وتم الكشف عن أدنى مستوى لمنطقة التثبيط عند التركيز 25 µg / ml ، حيث تكون مناطق التثبيط (0.33±4.67) *A.flavus*.

Introduction

Fungal contamination in health care facility had been the subject of many studies. These have observed that high percentages of hospital infections are caused by fungi, such as Candida spp and various species of *Aspergillus*, *Mucor*, and *Cladosporium* [1]. Apparently, Different fungi are causing infection; some of them are usually occurring infections while the others are rare [2]. *Aspergillus* is widely distributed in nature and found throughout the world, it seems to adapt to a wide range of environmental conditions and heat resistant conidia provide a good mechanism for dispersal. Disinfection process is removing pathogenic (kill or inhibiting growth) from materials or tools and prevent disease transmission, a process is less perfective than sterilization, which is Physics or chemical process include removal all microorganism, including spores (bacteria, fungi and virus) [3]. Nanoparticles exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology. Nanoparticles present a higher surface to volume ratio with decreasing size of nanoparticles. Specific surface area is relevant for catalytic reactivity and other related properties such as antifungal activity. Olive leaves extract (OLE) are rich in biophenols (BPs), such as oleuropein (Ole) which is the major active components in leaves , and its derivatives, verbascoside, ligstroside, tyrosol or hydroxytyrosol , as well as cafeic acid , p- coumaric acid , vanillic acid, vanillin, leteolin, diosmetin, rutin , luteolin-7- glucoside , apigenin-7glucoside and diosmetin-7-glucoside [4].These compounds with antifungal activity can be explored and used for the control of fungal diseases. However, the olive leaves extract and $AgNo_3$ lead to the formation of Silver nanoparticles (AgNPs) were used to inhibit or killing of pathogenic fungi. Many surveys have displayed the upshot effect of nanoparticles against some of the pathogenic fungi [5].The study aimed to biosynthesized of sliver nanoparticle from aqueous extract of olive leave and evaluate the effectiveness of the synthesis AgNPs against isolated fungi and comparison the effect with the effectiveness of Alcohol.

Materials and Methods

Samples collection:

From November in 2017 to February 2018, fifty samples were taken using sterile transport media swabs from various tools in the laboratory such as (loop, hood, incubator, refrigerator, cork borer, slide, and water bath.) from five hospitals in Baghdad.

Identification of fungi:

Different colonies of fungi were observed on (SDA) at 37°C for 5-10 day and appeared with different characteristic features such as (Colonies dense, Colonies dark green,Colonies compact white or yellow Colonies at first white to pale yellowish, Creamish- yellow in daylight, more grayish in darkness ,Light grayish ,Colony pale brownish-gray.Colony smooth radial furrows, whitish to cream-colored or grayish–brown). Through the duration of incubation, different fungal colonies were

subjected to microscopic and macroscopic to note their growth, mycelium nature and structure of hyphae. The growth of Filamentous fungal as mold and yeast on SDA, were sub cultured on separate SDA culture dishes. The incubation had been at 37°±1 C for one plate, while the others at 25°±1 C. The growth of the Pure culture for each yeast and mold colonies were examined to show their microscopic structures under magnification and clarified using mycological keys manuals.

Microscopic and macroscopic examination:

In this study, human pathogenic fungi were diagnosed according to [6]. This identification depends on the following:

1-Colony characteristics (color, consistency and topography).

2-Colony reverses (color, significant pigment).

3-Microscopic morphology (microconidia and macroconidia: their size, shape, arrangement, and hyphal structures).

Microscopic examination was made by the examination of many preparations from different areas of fungal growth mounted on a clean slide with lactophenol cotton blue stain to reveal spores which include large septate macroconidia and small, single-celled microconidia. The slide was gently heated in a spirit lamp in order to facilitate the staining and remove air bubbles (the excess stain was removed by a tissue paper) and then the cover slip was applied [7].

Frequency percentage [8]:

$$\text{Percentage frequency} = \frac{\text{Number of isolates of the same species}}{\text{Total number of isolates of all species}} \times 100$$

Occurrence Percentage [8]:

$$\text{Percentage of Occurrence} = \frac{\text{Number of samples that appeared to show one type}}{\text{Total number of samples}} \times 100$$

Aqueous extracts preparation:

Olive leaves were collected from olive trees in Baghdad University, Baghdad, Iraq. The leaves were washed for several clock time with distilled water to remove the particles of dust, then dried to remove the residual moisture and stinger it into small pieces. A measure of 100g from the small pieces of olive leaves was placed into the flask with 500ml of sterile distilled water and heated in the water bath at 60^{o}C for 2hr. Then the extract was cooled to room temperature and filtered for several times with Whitman no.1 filter paper by Buchner funnel. The aqueous solution of the extract was concentrated by using a rotary evaporator to remove the largest possible amount of water, and then put it in Petri dish at room temperature to dry. The stock solution of the extract was prepared with a concentration of 1 and1.5 mg/ml [9].

Biosynthesis of silver nanoparticles:

An aqueous solution (1mM) of silver nitrate (AgNO3) were prepared and used for the synthesis of AgNPs. 5 ml of plant extract added into 95 ml of a solution of 1mM silver nitrate for reduction into Ag+ ions. In a typical synthesis of silver (Ag) nanoparticles, the leaf extract (1.5 ml) was added to 30 ml of 10^{-3} M AgNO3 aqueous solution in a 250 ml Erlenmeyer flask and heated on a water bath at 75 °C for 60 min. Reduction of silver nitrate to silver ions was confirmed by the color change from colorless to brown .The formation of AgNPs confirmed by spectrophotometry determination. The fully reduced solution centrifuged at 5000 rpm for 30 min. The supernatant liquid discarded and the pellet obtained re-dispersed in deionized water. The centrifugation process repeated two to three times to wash off any absorbed substances on the surface of the AgNPs [10].

Characterization of AgNPs:

Ultraviolet-visible spectroscopy:

An Ultraviolet-Visible spectrophotometer (UV-Vis) refers to absorption spectroscopy. The samples were measured by UV-VIS double beam spectrophotometers from 300-600 Wave length.

Atomic force microscopy (AFM)

The surface morphology of the nanoparticles was visualized by Atomic force microscope (Veeco) under normal atmospheric conditions. The examined samples were dispersed on a small slide and explored on contact mode of the instrument [11].

Scanning electron microscopy (SEM)

The morphological characterization of the samples was done using JEOL Jsm6480 LV for SEM analysis. The samples were dispersed on a slide and then coated with platinum in an auto fine coater, after that the material was subjected to analysis [12].

Estimation of antifungal activity of silver nanoparticle:

Inoculum preparation

For the susceptibility testing of filamentous fungi, the inoculum prepared by serial dilutions method of the pathogenic fungi suspension

Agar Well Diffusion Method:

The different concentrations of silver nanoparticle (100, 75, 50 and25) mg/ml and suspension (1ml) of 10^{-1} and 10^{-3} dilution of pathogenic filamentous fungi were homogeneously mixed with SDA medium using the pour plate method in 6 cm Petri dishes. Then a well of (5mm) was made in the medium by using sterile cork borer. 100µl of each concentration of the silver nanoparticle was transferred into separate wells. Plates are incubated at 35±2°C for 1 day in *Aspergillus* spp. The diameter of the inhibition zone was recorded for each replicate and the average diameter was calculated [13].

Statistical Analysis

The Statistical Analysis System- [14] program was used to show effect of different factors in study parameters least significant difference –LSD test was used to significant compare between means in this study.

RESULTS and DISCUSSION

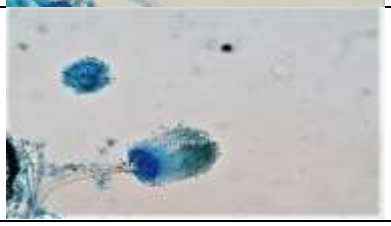
Indicator of Fungal Occurrence and Frequency:

In Tables- (1-4) showed that the highest occurrence and frequency of fungi is *Aspergillus niger* (96%) and (87.5%) respectively. This may be attributed to its high ability to produce enzymes and secondary metabolites which enable to exploit various minerals sources and resist wide ranges of environmental disinfectants [15].This result agreed with [16] showed that *Aspergillus niger* had highest occurrence and frequency of fungi .

Table 1-The table shows the Occurrence and Frequency of different fungi.

Genus	Occurrence %	Frequency%
1- *Aspergillus niger*	96%	87.5%
2- *Aspergillus flavus*	80%	62.5%
3- *Aspergillu fumigatus*	72%	53.5%
4-*Aspergillu nidulans*	48%	37.7%

Table 2-Showed the most important characteristics of *Aspergillus* spices on the SDA media and under microscope

No.	strains	View on SDA at 30 C after 5 days of incubation	Microscopic feature under (40 x)
1	***A.niger***		
2	***A. flavus***		
3	**A. *fumigatus***		
4	**A. *nidulans***		

Synthesis and Characterization of Silver Nanoparticles:

1- **Visual Observation and UV-Vis Spectral Study**:

After the first detection of silver nanoparticles presence in the chemical reaction vessel after the incubation periods which was the changeover of the reaction mixture from colorless to pale yellow, which indicated initial reduction, then to brownish yellow to light brown to deep brown - grey- with the time. UV-vies spectrophotometer was the next step to characterize the biosynthesized AgNPs. UV-Visible spectroscopy was used to examine the size and configuration of nanoparticles in aqueous suspension. Formation and stability of prepared AgNPs in sterile distilled water were approved by UV-Vis spectrophotometer in a range of 300 -800 nm of wavelength. Once olive leaves extract was mixed with AgNO3, the reduction response of Ag+ ion to Ag^{o} was observed by measuring the UV-Vis spectrum for the reaction media. (Figure-1) showed the recorded UV-Vis spectra after the completion of the reaction.

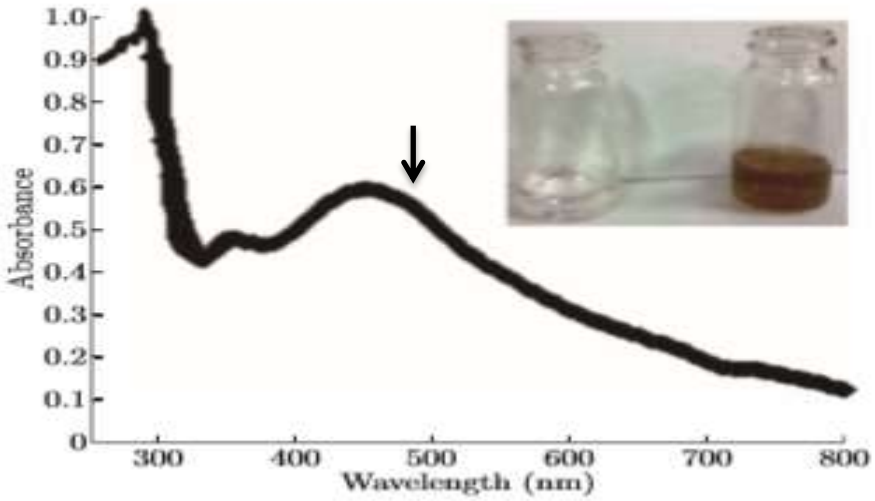


Figure 1-UV-Vis spectrophotometry of silver nanoparticles by olive leaf

Characterization by Scanning Electron Microscopy (SEM):

The scanning electron microscope was employed to analyze the shape of the silver nanoparticles that were synthesized by a green method. The surface-deposited silver nanoparticles are clearly seen at high magnification (100,000 kx) in the micrograph. SEM analysis shows that the olive leaf plant has tremendous capability to synthesize silver nanoparticles which were predominantly spherical in shape and were uniformly distributed with an average size little less than 50 nm (Figure-2).

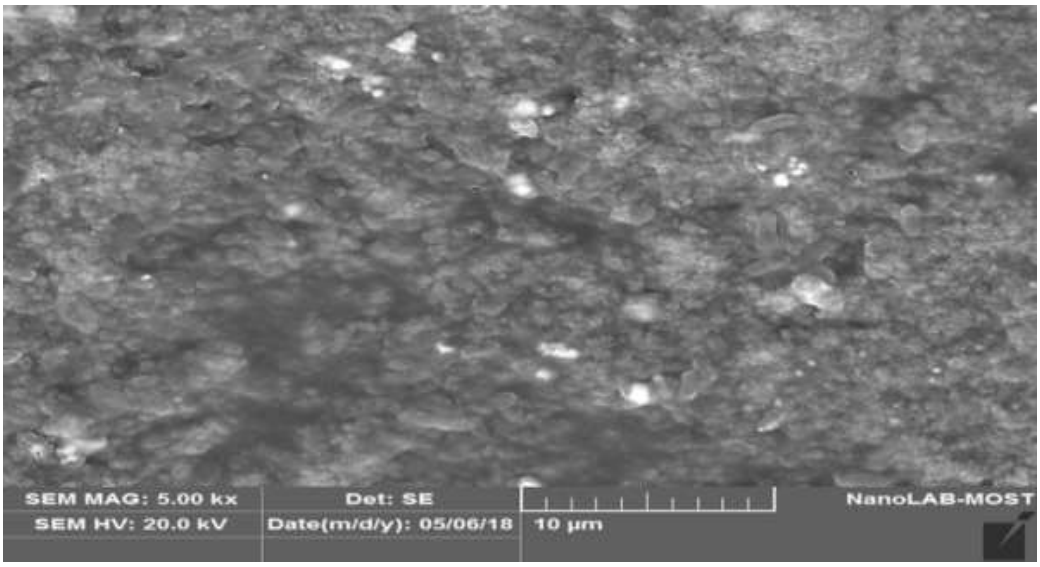


Figure 2-SEM image of AgNPs synthesized from olive leaves extract at 5.00 kx of magnification power.

Characterization by Atomic Force Microscopy (AFM)

Atomic force microscopy was used as a confirmatory technique to characterize the biosynthesis of AgNPs by detection their average diameter in addition to morphology in both two dimensions and three dimensions. The results obtained in this study showed that the biosynthesized AgNPs by olive leaf plant have average diameter 50.28 nm as shown in Table-3 and Figure-(3 a, b and c).

Table 3-The accumulation size of sliver Nanoparticles biosynthesis by olive leaf extract

Diameter(nm)<	Volume (%)	Cumulatio n(%)	Diameter(nm)<	Volume (%)	Cumulatio n(%)	Diameter(nm)<	Volume (%)	Cumulatio n(%)
30.00	0.70	0.70	65.00	4.90	20.98	100.00	8.39	64.34
35.00	0.70	1.40	70.00	6.99	27.97	105.00	12.59	76.92
40.00	2.10	3.50	75.00	5.59	33.57	110.00	3.50	80.42
45.00	1.40	4.90	80.00	6.99	40.56	115.00	4.90	85.31
50.00	4.20	9.09	85.00	2.10	42.66	120.00	6.29	91.61
55.00	2.10	11.19	90.00	8.39	51.05	125.00	8.39	100.00
60.00	4.90	16.08	95.00	4.90	55.94			

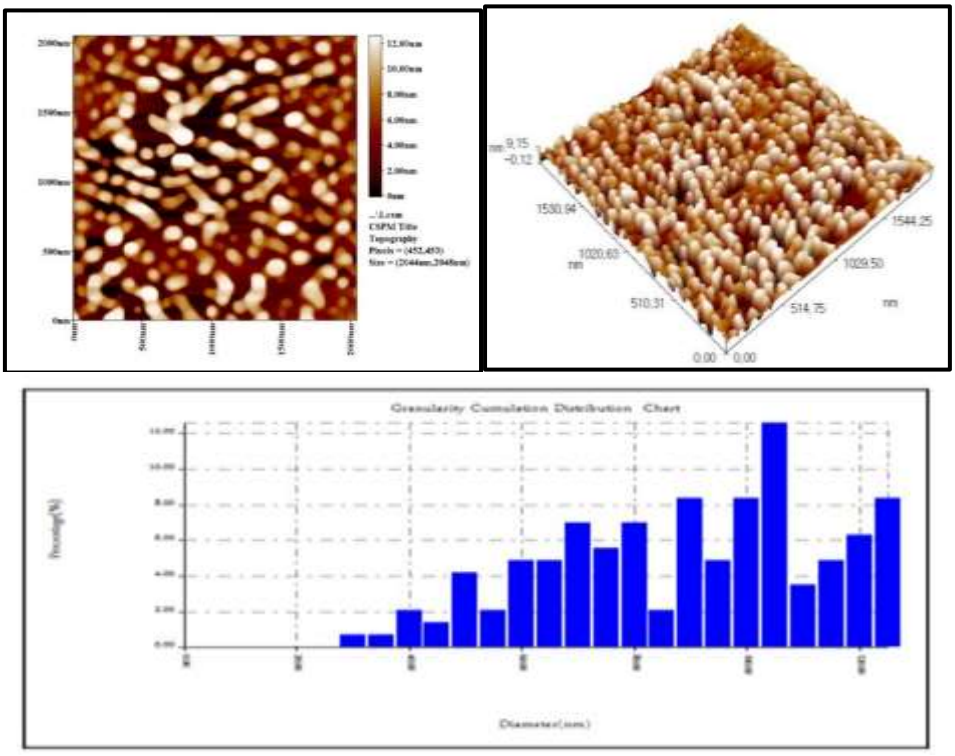


Figure 3-Biosynthesized AgNPs by olive leaf extract under AFM (a) 2D image of sliver Nanoparticales synthesis (b) 3D image of sliver nanoparticales synthesis (c) Granularity distribution chart of sliver nanoparticales synthesis

Antifungal activity of AgNPs

Table-4 showed the inhibition zone (mm) of fungal growth after treatment with AgNPs at the concentration 100,75 , 50and 25 µg/ml. The results showed that the maximum level of inhibition zone at (23.33 ± 4.41) for *A flavus* when the concentration of 100 µg/ml of AgNPs. While, the minimum level of inhibition zone at (6.00 ± 1.15) for *A.nidalus* when the concentration 25µg/ml of AgNPs.. It was noted that the inhibition level increase with the increasing of AgNPs concentration at all different cases. Alike studied by [17], they investagated a high reduction of fungal growth due to the higher AgNPs concentration. The inhibitory effect of AgNPs on the growth of fungi caused by [18]:

- Liberation of lipopolysaccharides.
- Changing of permeability of cell tissue layer.

- Membrane protein.
- Breakdown of the membrane potency due to the dissipation of the proton motive force.
- generation of free radicals responsible for the damage of the membrane.

Table 4-Growth inhibition zone of fungi by different Concentration of AgNPs

Fungi	*Concentration				LSD value
	100 mg/ml	**75mg/ml**	**50mg/ml**	**25mg/ml**	
A. niger	21.67 ± 2.02	12.67 ± 1.45	10.00 ± 1.15	7.67 ± 1.45	5.069 *
A. flavus	23.33 ± 4.41	20.00 ± 1.15	15.00 ± 2.88	7.67 ± 1.45	9.111 *
A. fumigatus	19.00 ± 2.08	12.33 ± 1.45	11.00 ± 2.08	8.00 ± 1.52	5.904 *
A. nidulans	20.00 ± 1.15	18.00 ± 1.15	14.33 ± 2.33	6.00 ± 1.15	5.011 *
(P<0.05).					

Antifungal activity of Alcohol (Ethyl alcohol):

The obtained results indicate that the highest level of inhibition zone was detected at the concentration of 100 µg/ml of Alcohol, where the inhibition zones are (12.33 ± 1.45) for *A.nidalus*, and the lowest level of inhibition zone was detected at the concentration 25µg/ml of Alcohol ,where the inhibition zones are (4.67 ± 0.33) for *A. flavus*. The results were summarized i Table- 5. Ethanol is well known as an inhibitor of the growth of microorganism. It has been reported to damage mitochondrial DNA in yeast cells [19], and to cause inactivation of some enzyme, such as hexokinase and dehydrogenase .Nevertheless, some strains of the Fungi appearance tolerance and can adapt to high concentrations of ethanol [20].Cell tissue layer has received extensive consideration as primary targets of ethanol stress. Many reports have suggested a relationship between the fatty acid physical composition of lipid membranes and ethanol stress tolerance [21]. This effect can be obtained by adding directly ethanol to the surfaces but at present, there is no available information about modeling the effect of ethanol on fungal outgrowth [22].

Table 5-Growth inhibition zone of fungi by different Concentration of Alcohol

Fungi	Concentration				LSD value
	100 mg/ml	**75mg/ml**	**50mg/ml**	**25mg/ml**	
A. niger	9.00 ± 2.08	6.33 ± 0.88	5.33 ± 0.67	5.00 ± 0.57	3.957 *
A. flavus	11.00 ± 2.08	8.33 ± 1.20	8.33 ± 1.20	4.67 ± 0.33	4. 415 *
A. fumigatus	9.00 ± 0.57	8.00 ± 1.15	7.67 ± 0.33	5.00 ± 0.57	2.369 *
A. nidulans	12.33 ± 1.45	10.67 ± 0.67	6.67 ± 0.67	5.33 ± 0.33	2.876 *

Conclusion

Many fungi isolated from laboratory tools, this result indicated the contamination in laboratory tools, Biosynthesized silver nanoparticles from aqueous extract of olive. Nanoparticles, aqueous extract of olive and alcohol used as antifungal, Silver nanoparticles more effective than aqueous extract of olive and alcohol that uses in sterilizing.

References

1. Perfect, J.R. and Casadevall, A. **2006.** Fungal molecular pathogenesis: what can it do and why do we need it?. p. 3–11. In: *Molecular principles of fungal pathogenesis*. Heitman, J., (ed.). ASM Press. Washington DC

2. Ryan, K.J. and Ray, C.G. **2010.** *Sherris Medical Microbiology*. 5th ed. The McGraw-Hill com. USA.

3. Rutala, W.A. and Weber, D.J. **2008.** the Healthcare infection control practices advisory committee (HICPAC). Guideline for disinfection and sterilization in healthcare facilities. Center of Disease Control and Prevention. Atlanta, G.A.

4. Farag, R.S., EL-Baroty, G.S. and Basuny, A.M. **2003.** Safety evaluation of olive phenolic compounds as natural antioxidants. *Int. J. Food Sci. Nutr*., **54**: 159-174

5. Verma, V. C., Kharwar, R. N. and Gange, A. C. **2010**. Biosynthesis of antimicrobial silver nanoparticles by the endophytic fungus Aspergillus clavatus. *J. Nanomdicine*., **5**(1): 33-40.

6. Tille, P. M. and Forbes, B.A. **2014**. *Baily & Scott's diagnostic microbiology* .thirteenth edition St.Louis, Missouri: Elsevier.

7. Aasi, S.R. and Al-Aaraji A.M. **2018**. The inhibitory effect of Trichoderma harzianum CA-07 crude extract against Trichophyton mentagrophyte and Microsporium canis. Iraqi Journal of Science. **59**(3B): 1387-1395.

8. Saleem, S.S., Alnakshabandie, W.M., Saadullah, A.A. **2018**. Fungal contamination of Azadi Teaching Hospital and Hevi Paediatric Hospital Environments, Duhok, Iraq. *Tikrit Journal of Pure Science*. **22**(6): 39-45

9. Nasir, G.A., Mohammed, A.K. and Samir, H.F. **2016**. Biosynthesis and characterization of silver nanoparticles using olive leaves extract and sorbitol. *Iraqi journal of biotechnology* .**15**(1).

10. Awwad, A. M., Salem, N.M. and Abdeen, A.O. **2013**. Green synthesis of silver nanoparticles using carob leaf extract and its antibacterial activity. *International J of Industrial Chemistry*., **4**: 29.

11. Oliveira, M., Ugarte, D., Zanchet, D. and Zarbin, A. **2005**. Influence of Synthetic Parameters on the Size, Structure, and Stability of Dodecanethiol-Stabilized Silver Nanoparticles. *Journal Colloidal Interface*., **292**: 429-435.

12. 12-Prasad, K. S.; Pathak, D., Patel, A. **2011**. Biogenic synthesis of silver nanoparticles using Nicotiana tobaccum leaf extract and study of their antimicrobial effect. *African Journal of Biotechnology*.,**10**(41): 81228130.

13. Sia, C. and Yim, H. a. L. C. **2010**. Commercial virgin coconut oil:assessment of antimicrobial potential. *Asian Journal of Food and Agro-Industry,* **3**: 567- 579.

14. SAS. **2012**. Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA.

15. Al Anni Soudad Abed Al Satar **1997**. Isolate and diagnoses of apportunistic fungi from Basrah hospitals with study of effected some disinfectans. MSc thesis Science College, Basrah University.

16. Reuf, D.R. **2000.**The epidemiology of hematogenous candidiasis caused by different candida species. *Clinical Infections Disease*, **24**: 1122-8.

17. Pulit J., Banach M., Szczygłowska R. and Bryk M. **2013**. Nanosilver against fungi. Silver nanoparticles as an effective biocidal factor. *Acta Biochemica Polonica*, 2013; **60**(4): 795-8.

18. Kim JS., Kuk E., Yu KN., Kim JH., Park SJ. **2007**. Antimicrobial effects of silver nanoparticles. *Nanomedicine: Nanotechnology, Biology, and Medicine*, **3**: 95-101

19. Ibeas, J.I. and Jimenez, J. **1997**. Mitochodrial DNA loss caused by ethanol in Saccharomyces flor yeasts. *Appl. Environ. Microbiol*. **63**: 7-12.

20. Alexandre, H., Rousseaux, I. and Charpentier, C. **1994**. Ethanol adaptation mechanisms in Saccharomyces cerevisiae. *Biotechnol. Appl. Biochem*. **20**: 173-183.

21. Farrag, A., Ismail, M.A., Abdel-Razek, K.A. and Ali, A.A. **2012.** In vitro antifungal effects of some chemotherapeutic agents against fungi commonly isolated from repeat breeder animals. *Journal of Basic & Applied Mycology*. **3**: 13-19.

22. El-Said, A.H.M. and El-Hady, G. **2014**. Effect of moisture contents on the biodiversity of fungi contaminating Cuminum cyminum and Pimpinella anisum seeds under storage periods and amylolytic activity of fungal isolates. *Int. J. Curr. Microbiol. App. Sci*. **3**(3): 969-991.