Study of the Antifungal Effects of Copper-based Pigments and Synthesized Nanomaterial on Mural Painting-deteriorated Fungi in the Egyptian Museum in Tahrir

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Abstract

Seven fungal strains, namely: Aspergillus terreus, A. clavatus, A. niger, A. humicola, A. sydowii, Paecilomyces variotii and Fusarium oxyspourm were isolated from Stelae dates back to the 19th Dynasty of the New Kingdom of the reign of King Sity in ancient Egypt, respectively. The isolated fungi were identified by studying their macro and micro-morphology. Furthermore, nanogel, nanolime and nonosilver were tested to study their ability to inhibit the fungal growth of the isolated fungi. The potent fungus, a. terreus, which showed best result in nanosilver biosynthesis Aspergillus clavatus (25mm) > Aspergillus niger (24mm) > Aspergillus sydowii (22mm) > Paecilomyces variotii (20mm) and Fusarium oxysporm (20mm) > Aspergillus terreus (14mm) > Aspergillus humicola (12mm), was further identified by the molecular technique (18SrRNA). Nanosilver. On the other hand, nanolime followed nanosilver in its antifungal effect against isolated fungal strains and its activities followed the order: Aspergillus niger (22mm). Different nanomaterials (nanogel, nanolime and nanosilver) were prepared either chemically followed by nanogel exhibited the highest antifungal activities against the isolated fungi whereas nanolime exhibited weak antifungal activity. The effect of archaeological pigments (Malachite, Azurite, Egyptian green and blue) on the isolated fungi as antifungal agents was also investigated. It has been found that Azurite and malachite showed a considerable effect more than the Egyptian green and Egyptian blue. The hydrolysis of animal glue, binding material, was also studied by cultivating the isolated fungi in broth medium containing animal glue as nitrogen source and the proteolytic activity of their culture filtrate has been assayed.

Key Words: Stelae, Pigment, Fungi, Nanomaterials, Animal glue.

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1. Introduction

Stelae, is a piece of carved stone of rectangular shape mostly of round top and usually leave their back surface without glazing (Megahid, 2001). Funerary stelae were usually inscribed with the name and title of the deceased, along with images or hieroglyphs (Abdel-Naby, 2004). Copper-based mineral, one of the great variety of inorganic pigments used in mural paintings, are often affected by degradation leading to colour change or darkening. Several environmental factors such as humidity, temperature, harmful gases, organic bending media, microbial activities, alkali, salts or catalytic activities of elements could be responsible for the alteration of the colour and stability of Stelae (Robert and Zieman, 2014). Potential risks for the degradation of these objects may take place by different microorganisms. The microbial activities in museum galleries and storage areas depend on other synergetic factors, i.e., atmospheric conditions, the nature of artifacts, the location; materials help in microbial attack and physicochemical degradation processes. Under favorable conditions (humidity, temperature, light and substrate), these airborne microbes could grow and causing irreversible deterioration to the artifacts (Ciferri, 1999). In particular, wall paintings, such as murals or frescos, are usually poor in organic substances, and the development of the heterotrophic microorganisms is conditioned not only by microclimatic factors, but also by the biological pollutants that are presented in the air that arise from the spread of biodeterioration organic substances that represent sources of nutrients for many microorganisms. Such artifacts can suffer from degradation, and the problem of biodeterioration that arises when live cells of biodeteriogenic microorganisms are deposited on the surfaces. Under favorable conditions of temperature and humidity, heterotrophic microorganisms can be sufficiently able to develop and multiply, such as the bacteria and fungi that can be found in minimal dust deposits or organic residues that are generated by the primary and secondary autotrophic colonizers (Ruga et al. 2015). To overcome the irreversible deterioration, it is important to monitor the microbial quality in the air of environment surrounding the artifact. Alterations of paintings were mainly depending on their chemical composition. In general, copper-based pigments are more susceptible to acids than iron based pigments (Strzelczy, 1981). Fungal deterioration of Stelae is mainly physical and chemical in nature. In physical deterioration, the fungal hyphae are growing inside the paints layer causing mechanical destruction and by further growth, the smooth surface of the painting will modify and the painting became rough (Agrawal et al., 1988) and these effects could lead to exfoliation, cracking and loss of pigment of the paints (Claudio et al., 2006). On the other hand, in chemical detereioration the produced enzymes from fungi that could uptake wall paintings as carbon source were responsible for the excretion of acids and pigments that can damage and stain the painting surface (Garg et al., 1995). Nanomaterials have extremely small size and exhibited high chemical activities and achieved more progress in cleaning of art works especially for removing of some resins from mural paintings and marble protecting them from deterioration (Grassi et al., 2009). The first application of nanoscience in conservation of artifacts was dates back to the end of the year 1980 in Florence (Italy) in the cleaning of the wall paintings of Brancacci Chapel. The cleaning was irreversible and delicate intervention involving the removal of unwanted materials layer by layer has been achieved. The cleaning of Brancacci Chapel has been achieved by using oil in water microemulsion of dodecane nanodroplets stabilized in water by surfactant that used in the removal of wax spots from the surface of the mural painting

(Baglioni et al., 2015). Microemulsions are thermodynamically stable and could be used in different environmental conditions without forming two separate layers (organic and aqueous). Carretti and Dei (2004) studied the preparation of the gel acrylic amide polyacrylic acid that exhibited a wide role in cleaning. Natali et al. (2011) investigated the preparation of gel (peelable) that contained different organic solvents and used in the removal of dust from the artwork. Nanolime, nanoparticles of calcium hydroxide, was recently synthesized and it exhibited stability in aqueous media that prepared by selecting suitable solvent (Armada and Hirest, 2012; Bagglioni et al., 2014). Nanolime was considers as a prolific tool for conservation of wall paintings because their small particles could penetrate the layers of paints and this mechanism has been performed with the aid of dispersing solvent (Giorgi et al., 2010). Dei (2006) investigated the potential use of nanolime in the consolidation of stones and mural paintings. Boglioni (2008) reported that nanolime showed an efficient role in the consolidation of mural paintings and explaining its stability in different conditions and its ability to improve the mechanical properties of the treated layers and decreasing water absorption (hydrophobicity). Silver nanoparticles (AgNPs), due to their exclusive physical and chemical properties, are widely used in different fields, i.e., medical, health care, consumer, food and industrial applications. These applications include optical, electrical, and thermal, high electrical conductivity and biological properties as well (Mukherjee et al., 2001; Li et al., 2010; Gurunathan et al., 2015]. Due to their unique characteristics, they have been used for many applications as antibacterial agents, in industrial, household, and healthcare-related products as well as anticancer (Chernousova and Epple, 2013). Different binding media such as Arabic gum, animal glue, egg yolk as well as bees wax were mixed with pigments like hematite, limonite, azurite blue as well as malachite in ancient Egyptian paintings (Newman and Serpico, 2000). Animal glue is an animal protein composed mainly of amino acids that could be produced by the aid of microbial collagenase enzyme (Chadefaux et al., 2009). Thus paintings having animal glue as binding agent when subjected to microbial attack lose its cohesiveness from plaster layer and stone support (Goshev et al., 2005). Ancient paintings were based on copper have been mostly used. Copper has been used as an antimicrobial means since ancient times, before the discovering of microorganisms in the 19th century, and gave successful results when used by physicians in surgical wounds in the early 1800s. It has been known that the first time copper was used in medicine as a biocide was by an Egyptian doctor recorded in the Smith Papyrus around 2600 and 2200 BC (Grass et al., 2011). The Phoenicians have been also used copper and silver bottles to keep wine, water, and vinegar and in the 1st World War, copper was used to prevent wound contamination (Gabbay et al., 2006). Nowadays, copper and its alloys are extensively used as chemical biocides for medical and non-medical purposes, i.e., bactericides to act as self-disinfectants in paints, purify water distribution systems, Legionella in hospitals (Borkow and Gabbay, 2004), fungicide in agriculture to protect some plants such as coffee, tea, citrus and cocoa from fungal leaf diseases (Cervantes and Gutierrez-Corona, 1994; Kiaune and Singhasemanon, 2011), and as an active ingredient in many pesticide formulations mainly after the tributyltin was banned in the late 1980s (Kiaune and Singhasemanon, 2011). This study has been undertaken with the aim of isolating fungi from deteriorated Stelae and identifying them. Also this work has been dealt with the synthesis of some nanomaterials to be used as antifungal

agents and cleaning materials. The effect of fungi on the painting pigments and the binding material (animal glue was also studied).

2. Materials and Methods

2.1 The Object and Sampling

A Stela dates back to the he 19th Dynasty of the reign of King Sity from the New Kingdom was selected for this study. It is located in the Egyptian Museum in Tahrir in the storage magazine number (SR 4\14199) (JE36850). It was discovered in 1904 in the excavations of the City of Kom Medinet Ghurab (Moeris) in Fayoum (Egypt). The Stela dimentions are length 32 cm - width 26 cm and thickness 5. The Stela suffers from the following deterioration phenomena (see Figures 1). Biological samples for the study were taken from the mural painting (Stela) in the Egyptian museum. The Stela suffer from the discoloration and growth some microorganism on surface. Samples were undertaken with the help of a sterile glass-fiber brush and microtubes (for cultivation).

Figure 1: A, surface of the Stela of Iy and B, back of the Stela.

2-2 fungal isolation

Fungi on the Stelae were isolated using the following method: sterile cotton swabs were wiped across fungal colonies then transferred to the laboratory in sterile tubes and used for fungal isolation. All samples were transferred to the laboratory at the same day of collection and immediately processed. Each swab, wiped across fungal colonies on the Stelae, was immersed in a sterile glass vial containing 5ml of sterile distilled water and shaken for 2 hours on a reciprocal shaker. Aliquots (200µl) of spore suspension were spread on 15cm Petri dishes (3 plates per sample) containing potato-dextrose agar medium (PDA), comprising: Potato infusion (200g/L), Dextrose (20g/l) and agar (20g/L). This medium has been supplemented with the antibacterial agent (Streptomycin, 0.1%) and Rose Bengal to limit the fungal growth. Plates were incubated for 7 to 14 days at 30°C in the dark and the single colonies were picked up and used to inoculate potato dextrose agar slants.

2-3 Fungal Identification

The most biodeterioration active fungi isolates (number of isolates) were identified morphologically and biochemically. The identification of mould isolates were carried out on the basis of their macro and microscopically characteristic sporulation according to the keys of **Gilman (1957), Barnett** and **Hunter (1986**), **Domsch et al., (2007) and Samson et al., (2010).** The identification of fungal isolates was carried out in the Microbial Chemistry Department, National Research Centre, and Egypt.

2-4 Molecular identification the fungal strain

The molecular identification of potent fungus used in the biosynthesis of silver nanoparticles has been accomplished according to a molecular biological protocol by DNA isolation, amplification (PCR) and sequencing of the ITS region. The primers ITS2(GCTGCGTTCTTCATCGATGC)and ITS3 (GCATCGATGAAGAACGCAGC) were used for PCR while ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used for sequencing. The purification of the PCR products was carried to remove unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied Biosystems, USA). Sequencing products were resolved on an applied Biosystems model 3730XL automated DNA sequencing system (AppliedBiosystems, USA). Candida sp. was used as control.

2-5 Preparation of nanomaterial for cleaning the Stelae.

2-5-1 Nanogel - O\W microemulsion

The O/W Microemulsion1 was prepared according to Baglioni et al., (2012). This microemulsion is very important in cleaning fresco paintings by dispersing a given amount of Triton 100 (2.45ml) in an aqueous solution containing 46.3ml of water stirred using the magnetic stirrer with 535 -540 RPM (round per minute) for one hour. The preparation was prepared according to published report. In brief, ammonium carbonate was gradually added while stirring at 45°C (1.05mg). The system which is initially opalescent suddenly becomes limpid after a few minutes. Next the dispersed phase p-xylene (0.20ml) was added the stirring process is continuous until the solution is totally transparent **(Baglioni et al., 2012).**

2-5-2 Calcium hydroxide $Ca(OH)_2$ Nano (calosil – micro)

Two nanolime synthesis methods are performed. Method A According to method A, to obtain 2.20 g of $Ca(OH)_2$, two initial aqueous solutions of 100mL containing 3.33 g of CaCl2 (corresponding to 0.3 mol/L of $CaCl_2$) and 2.40 g of NaOH (corresponding to 0.6 mol/L of NaOH) respectively are prepared. Maintaining a thermal bath at a temperature of about 90°C, the NaOH alkaline solution is added drop by drop (speed 4 mL/min) into the $CaCl_2$ one (S0 sample). This procedure requires about 40 minutes (including the time to prepare the initial solutions and the mixing time). Method B According to method B, to obtain 2.20g of $Ca(OH)_2$, Triton X100 is previously added to calcium chloride initial aqueous solution, which is later mixed simultaneously to the aqueous sodium hydroxide one, at the fixed temperature of 90°C. In particular, ST1, ST2 ST3 and ST4 samples are considered, characterized by different surfactant contents, equal to 0.1 g, 0.2g, 0.4g and 1.0g respectively. This procedure requires about 10 minutes (including the time to prepare the initial solutions and the mixing

time). Both in case of methods A and B, two distinct phases are observed: a limpid supernatant solution and a white precipitated phase. After five washings, necessary to remove the NaCl produced, water is partially removed and substituted by an equal alcohol content, obtaining a water/alcohol ratio (W/A) of 0.1. All the suspensions are characterized by a final concentration of about 10mg/mL (**Danielea and Taglier, 2012).**

2-5-3 Biosynthesis of silver nanoparticles

A-Screening and biosynthesis of silver nanoparticles

All the isolated fungi were screened for their ability to biosynthesize silver nanoparticles by cultivating them on potato dextrose broth (g/l): Potato infusion (from 200g potato), dextrose (20), pH (6) and 1000ml distilled water. The cultures were incubated on a rotary shaker (150rpm) for 7 days at 30^{o}C. The fungus Aspergillus terreus which isolated from the powder of the Egyptian blue paint given from the stelea of Egyptian museum (Egypt-El Tahrir). So this Strain was selected for the biosynthesis of sliver nanoparticles according to **(Xue et al., 2016)**. Ten ml culture filtrate of the fungus was mixed with 50 ml of 1 mM silver nitrate solution in 250 ml conical flask and agitated at room temperature. After 72 hours of time interval culture filtrate and silver nitrate solutions turned into reddish brown due to reduction of silver nitrate to silver nanoparticle (metal).

B-Characterization of silver nanoparticles

Characterization of synthesized silver nanoparticles

UV- Visible spectroscopy

The bioreduction of silver nitrate ($AgNO_3$) to Ag-NPs and were monitored periodically by measuring the maximum absorbance by UV–VIS spectroscopy (Shimazu 2401PC) **(Vahabi et at 2011).** A UV–VIS spectrograph of the silver nanoparticles was recorded by using a quartz cuvette with water as reference. The UV–VIS spectrometric readings were recorded at a scanning speed of 200–800 nm **(Magdi et al., 2014)**.

X-Ray diffraction studies

Powdered sample of silver and gold nanoparticles was used for X-ray diffraction; The Coherently diffracting Crystallography domain size of the Silver nano particle was calculated from the width of the XRD peaks using scherrer formula. X-ray diffraction (XRD) measurements of Aspergillus sp. reduced silver and gold nanoparticles were carried out on drop-coated films of the respective solutions onto glass substrates by a Phillips PW 1830 instrument operating at a voltage of 40 kV with Cu Ka radiation **(Saad et al., 2017)**.

Transmittance Electron Microscope (TEM) investigation

TEM analysis of Ag-NPs has been evaluated using JEOL model 1200 EX electron microscope. TEM samples were prepared by placing a drop of the suspension of Ag-NPs solutions on carbon-coated copper grids and allowing water to evaporate. The samples on the grids were allowed to dry for 4 min. The shape and size of silver nanoparticles biosynthesized by Aspergillus terreus has been evaluated by TEM **(Abdel-Aziz et al., 2014).**

Antimicrobial activity

The previously prepared nanosamples (nanosilver, nanolime and nanogel) were tested against the Stella-isolated fungal strains (Aspergillus terreus, Aspergillus sydowii, Paecilomyces variotii, Aspergillus clavatus, Fusarium oxyspourm, Aspergillus niger and Aspergillus

humicola). The antifungal activities of different samples were investigated by the agar cup plate method. A Potato-Dextrose agar plates seeded by 0.1ml the fungal inoculum (10^6-10^7 CFU) was used to evaluate the antifungal activities. Then a hole (1cm diameter) was made in media by gel cutter (Cork borer) in sterile condition. Then one drop of melted agar was poured into hole and allowed to solidify to make a base layer. After that specific amount of tested sample (0.1 ml) was poured into the hole. Then plates were kept at low temperature (4°C) for 2-4 hours to allow maximum diffusion. The plates were then incubated at 37°C for 24 hours for bacteria and at 30ºC for 48 hours in upright position to allow maximum growth of the organisms. The antimicrobial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter (mm). The experiment was carried out more than once and mean of reading was recorded **(Abdel-Aziz et al., 2014; Barry, 1976**).

Effect of the isolated fungi on different Pigments

The effect of the seven fungi previously isolated from ancient stelae from the Egyptian Museum El Tahrir on different paints with and without animal glue has been studied. Potato dextrose agar plates were used in this study. Firstly, the plates were inoculated with the isolated fungi by spreading them on the top of the plates using sterile swab then the paints were spread on the surface of inoculated plates. The plated were incubated at 30ºC for different incubation periods (1, 2 and 3days). The used fungi are Aspergillus terreus, Aspergillus sydowii, Paecilomyces sp., Aspergillus clavatus, Fusarium oxyspourm, Aspergillus niger and Aspergillus humicola.

Growth of isolated fungal strains on medium containing animal glue

Animal glue was used as a nitrogen source for the growth of Aspergillus terreus, Aspergillus sydowii, Paecilomyces variotii, Aspergillus clavatus, Fusarium oxyspourm, Aspergillus niger and Aspergillus humicola that isolated from Stelae These fungi were cultivated on Saboraoud-dextrose-glue broth medium with glue of a concentration of 2%. Erlenmeyer flasks of 250ml-volume each having 50ml of this medium and each was inoculated with 10% sporesuspension from each fungal strain under investigation **(Hamdy, 2008).** The flasks were incubated at 30ºC for 7days on a rotary shaker (150rpm). Mycelia were separated from the whole culture medium by centrifugation at 5000rpm for 20min at 4ºC (Centerion, cooling centriguge, UK). Mycelia were dried at 80ºC for 24h, using pre-weighed nitrocellulose membranes, and the weight of each fungus was recorded. The degradation of the glue by the tested fungi were investigated either by measuring the dry weight or proteolytic activity for each fungus.

Proteolytic activity measurement

The activity of alkaline protease in the cell-free supernatant was measured by modified method of **Takami et al. (1989)** alkaline protease activity was determined by using casein as a substrate at a concentration of 1% w/v in 50mM Glycine-NaOH buffer (pH 9). The assay was carried out routinely in a mixture containing 0.5ml of a suitably diluted enzyme solution and 2.5 ml casein solution. After incubation for 1 hour at 30C, the reaction was terminated by the addition of 2.5ml of 0.44M trichloroacetic acid (TCA) solution. After 10min the mixture was centrifuged at 8000 rpm for 10min. An aliquot of 0.5ml of supernatant was mixed with 2.5ml of 0.5M Na_2CO_3 and 0.5ml of Folin-Ciocalteu's phenol solution and kept for 30min at room temperature. The optical densities of the solutions were determined with respect to sample blanks at 660nm.

3- Results and discussion

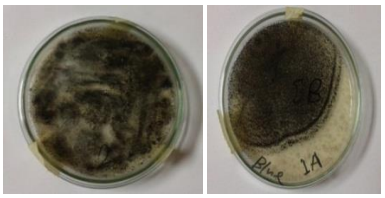
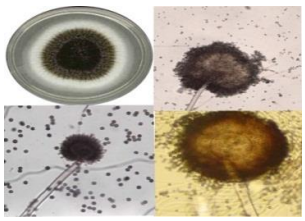
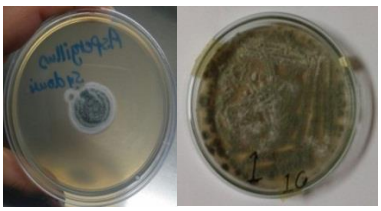
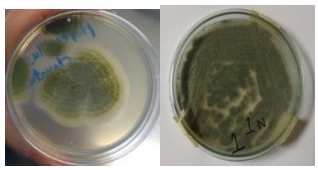
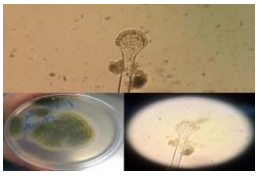
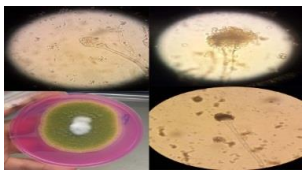
3-1 fungal isolation

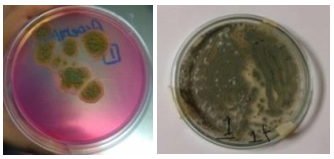
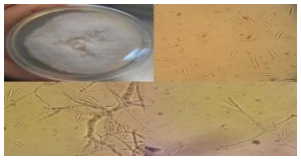
Seven fungal species were isolated from Selae using potato dextrose agar medium supplanted with the antifungal streptomycin (0.2%) and a group of plates were supplemented with Rose Bengal, to control fungal growth and the other without Rose Bengal. (Table 1) showed the isolated fungi grown on the PDA plates.

3-2 fungal identification

The isolated fungi were manually identified according to according to the keys of **Gilman (1957), Barnett** and **Hunter (1986), Domsch et al (2007)** and **Samson et al (2010),** the identification of fungal isolates was carried out the microbial Chemistry Department, National Research Center, Egypt. This identification technique depends mainly on the studying of the morphology of fungal strain either on the cultivation medium or under light microscope as well as the studying of some biochemical characteristics.

Table 1: Fungal growth on potato dextrose agar (PDA) plates and their appearance under light microscope.

Fungus	Photos of fungal growth on agar plates	Picture under microscope
Aspergillus niger		
Aspergillus sydowi		
Aspergillus clavatus		
Aspergillus humicola		
Aspergillus terreus		

Paecilomyce variotii		
Fusarium Oxysoprum		

3-3 Nanogel O\W microemulsion

The average modern infrared instrument records spectra from 400-4000 cm^{-1}. The data confirmed with FT-IR spectra (is a 4100 Jasco-Japan) which are given in the (fig 2). This has characteristic peaks of Nano gel shows 296, 744.77, 1247, 1187, 1094, 3400 this groups frequencies help to characterize a compound, and the combination of the bands associated with these group frequencies and the skeletal frequencies are used to identify a specific compound. FTIR which measured the function groups of Nano gel have confirmed the presence of functional groups, show the IR to microemulsion, N-H 3323 cm^{-1}, C=C 2107 cm^{-1} and others bending 1247, 1187, 1094 cm^{-1}. **El-Sheikh et al. (2017)** when preparing nanogel, they got approximately the same IR results.

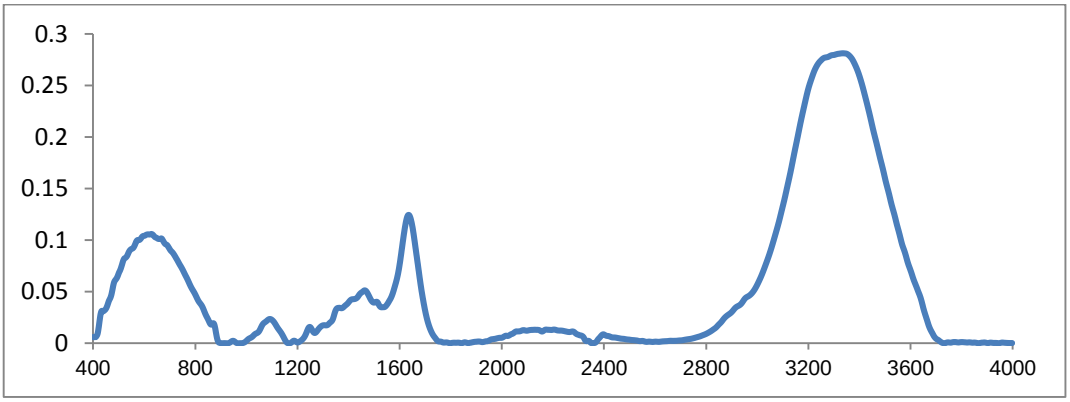


Figure2. Infra-red chromatogram (IR) of nanogel –microemulsion

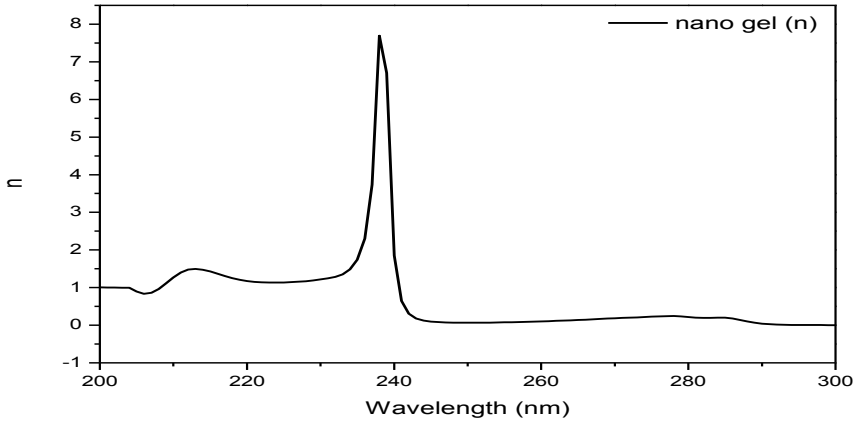


Figure3: UV to nano-gel at 240 cm^{-1}.

3-4 Calcium hydroxide $Ca(OH)_2$nano (calosil-micro)

The average modern infrared instrument records spectra from 400-4000 cm-1. The data confirmed with FT-IR spectra which are given in (Fig 4) this has characteristic peaks of Nano lime shows 844.77, 1172.85, 1638.06, 2400, 2313.52, 3400 cm^{-1} this groups frequencies help to characterize a compound, and the combination of the bands associated with these group frequencies and the skeletal frequencies are used to identify a specific compound. FTIR which measured the function groups of Nano lime have confirmed the presence of functional groups. These findings are similar to that obtained by **Reddy Subramanian (2016).**

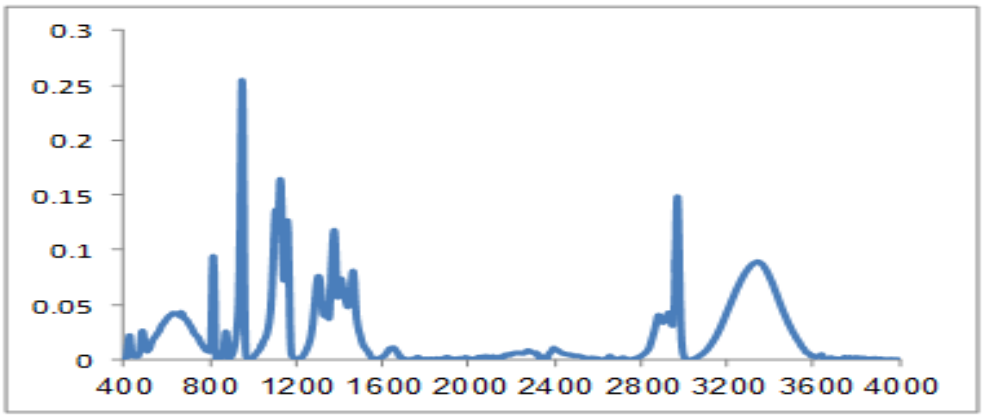


Figure4. Infra-Red chromatogram (IR) of nano lime.

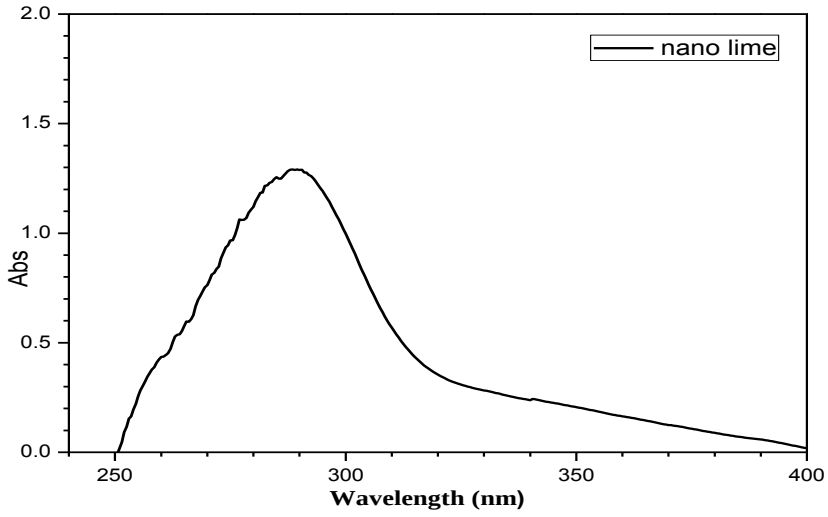


Figure 5: UV to nanolime at 290 cm^{-1}.

Color change and UV-VIS absorbance

The addition of the fungal culture filtrate to either silver nitrate solution resulted in the formation of reddish brown colour. Results in (Fig 6). showed the formation of reddish brown colour indicating the formation of silver nanoparticles. Several investigations have been focused on the biosynthesis of silver nanoparticles from different microorganisms including bacteria, fungi and yeast **(Castro-Longoria et al., 2011; Klaus-Joerger et al., 2001; Ahmad**

et al., 2003; Mandal et al., 2006; Lin et al., 2005). The produced nanoparticles were subjected to Uv-Vis spectrophotometric measurements (Fig 7). It has been found that the produced solutions of Ag-NPs (brown) exhibited maximum absorbance at 420nn. Silver nanoparticles were biosynthesized by using the fungus Arthroderma fulvum showed a change of coulour of clear (AgNO3 solution to reddish brown (AgNPs and exhibited maximum UV/Vis absorbance at 420 nm **(Xue et al., 2016).** Silver nanoparticles biosynthesized by Pleurotus ostteatus had a reddish brown colour and showed a maximum absorbance at 420-440nm **(Devika et al., 2012).**

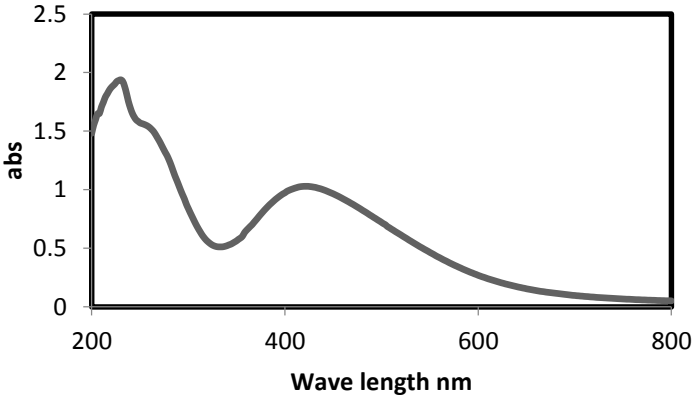


Figure 6: Color change of silver nitrate (colourless) to silver nanoparticles (reddish brown) by Aspergillus terreus SEHM1.

Figure 7: The UV/Vis spectra of the silver (Ag-NPs) biosynthesized by Aspergillus terreus SEHM1.

Studying the structure properties of Ag-NPs by XRD

The XRD consider as well as TEM or SEM as the most important technique to study structural properties of the prepared nanomaterials, the prepared Ag-NPs were examined using the XRD diffraction pattern. (Fig 8) Represent the XRD result of Ag nanoparticles. Fig. displayed the characteristic peaks of metallic Ag found at 37.5°, 43.4°and 63.8° corresponding to the crystallographic planes (1 1 1), (0 0 2), and (0 2 2) of Ag, respectively, creates a characteristic of crystalline metallic Ag phase **(Suh et al., 1988).**

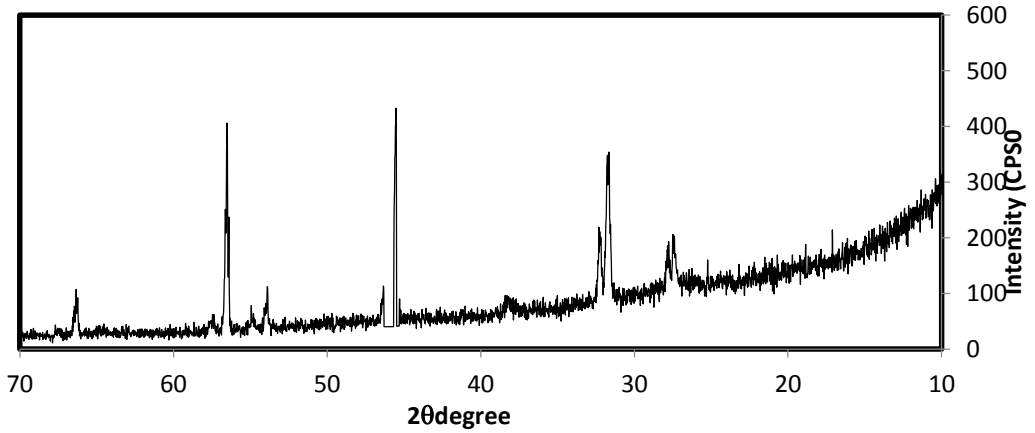


Figure 8: show the XRD to nanosilver.

Transmittance electron microscopy (TEM) studies proved that the biosynthesized silver nanoparticles (Ag-NPs) exhibited average sizes from 5-40nm. Silver nanoparticles with different sizes were biologically synthesized by different fungal strains. Silver nanoparticles were iosynthesized using the fungus Rhizopus stolonifer giving rise to nanoparticles with round shape and with a size of about 6nm **(AbdelRahman et al., 2017)**. Moreover, silver nanoparticles biosynthesized by Aspergillus niger exhibited particle size around 20-55nm **(Ninganagouda et al., 2014)**.

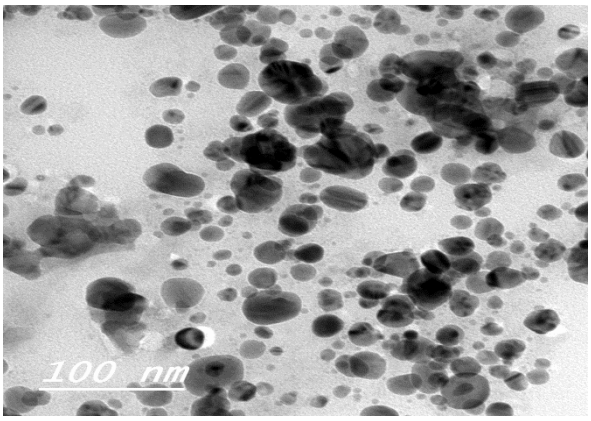


Figure9. Transmittance Electron Microscopy (TEM) of Silver nanoparticles (AgNPs) biosynthesized by Aspergillus terreus SEHM1 culture filtrate.

3-5 Molecular identification of Aspergillus terreus

Nucleotide sequence of 603bp (Fig 10) of the whole 18S rRNA gene of the fungal sp. isolate SEHM1 was determined in both strands. Blast search revealed 100% similarity to Aspergillus terreus strain PAS3 (Acc. no. KY806124.1). The AB1 photograph and phylogentic tree of this fungus were also constructed. Several investigations used the molecular and biological technique (18S rRNA) in the identification of isolated fungi. The fungus was identified as Aspergillus terreus SEHM1with the Gene Bank accession number (MH712038) and PubMed link: https://www.ncbi.nlm.nih.gov/nuccore/MH712038

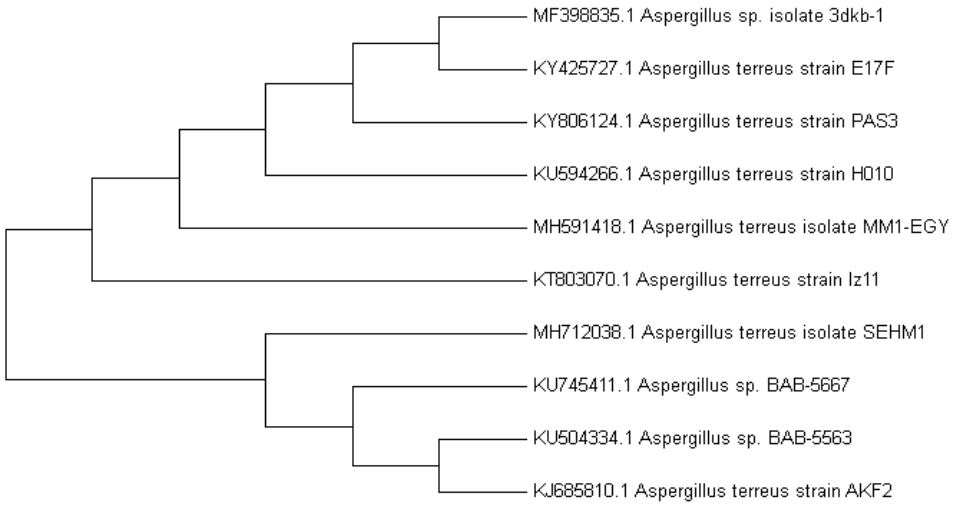


Figure 10: Phylogenetic trees showing relationship of strain Aspergillus terreus isolate SEHM1 with other related fungal species retrieved from Gen Bank based on their sequence homologies of 18S rRNA.

3-6 Antifungal activities of prepared nanomaterial on isolated fungal strains

The previously prepared nanopartices, i.e. nanogel, nanolime and nanosilver were tested for their ability to inhibit the growth of the isolated fungi. This study has been performed by using the cup plate technique and the clear zones appeared around the cup were put in our consideration as positive results. Results in (Table 2) and (Fig 11) revealed that nanosilver exhibited the highest antifungal activities against all isolated fungi under study and the activities against these fungi followed the order: Aspergillus clavatus (25mm) > Aspergillus niger (24mm) > Aspergillus sydowii (22mm) > Paecilomyces variotii (20mm) and Fusarium oxyspourm (20mm) > Aspergillus terreus (14mm) > Aspergillus humicola (12mm). On the other hand, nanolime followed nanosilver in its antifungal effect against isolated fungal strains and its activities followed the order: Aspergillus niger (22mm) > Aspergillus humicoloa (20mm) and Fusarium oxysporum (20mm) > Aspergillus clavatus (17mm) and Aspergillus sydowii (17) > Aspergillus terreus (12mm). Nanogel showed the lowest effect of all tested nano-materials. Nonogel didn't show any antifungal activities against Aspergillus terreus, Paecilomyces variotii, Aspergillus clavatus and Aspergillus humicola. Nanolime showed antifungal activities against Aspergillus sydowii (15mm), Aspergillus niger (15mm) and Fusarium oxyspourm (13mm). Generally, the most affect fungus by all nanomaterials was Aspergillus niger and the most resistant fungus to all nanomaterials was Aspergillus terreus. Silver nanoparticles (AgNPs) have been used as antimicrobial agent for a long time. Recently, Silver nanoparticles (AgNPs) have been used as antimicrobial coating for stone heritage. In cultural heritage, the silver nanoparticles have been grafted to Italian Serena sandstone surfaces to inhibit bacterial growth **(Bellissima et al., 2014)**. The nanoparticles were functionalized through the condensation of a silane precursor (tetraethylorthosilicate, TEOS) on the surface of silver nanoparticles, and showed an effectiveness ranging from 50 to 80% in reducing cell growth. **Aflori et al. (2013)** developed two silsesquioxane-based hybrid nanocomposites with methacrylate units modified with titania and/or silver nanoparticles to be used as antibacterial and antifungal coatings. Silver nanoparticles were biosynthesized from plant leaf extract (Carrillo-González et al., 2016) and studied their efficiency in controlling bacteria and fungi in vitro as well as on different types of stones (stucco, basalt and calcite) widely applied to cultural heritage. They detected the utilization of silver nanoparticles as promising antimicrobial tools for cultural heritage conservation and they found that the activity was highly dependent on the selected doses. Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell. There is formation of ‘pits’ on the cell surface, and there is accumulation of the nanoparticles on the cell surface **[Sondi and Salopek-Sondi, 2004]**. The formation of free radicals by the silver nanoparticles may be considered to be another mechanism by which the cells die. There have been electron spin resonance spectroscopy studies that suggested that there is formation of free radicals by the silver nanoparticles when in contact with the bacteria, and these free radicals have the ability to damage the cell membrane and make it porous which can ultimately lead to cell death **[Danilcauk et al., 2006; Kim et al., 2007]**. Interaction of silver nanoparticles with the thiol groups of many enzymes and thus

inhibiting the vitality of microbial cells **(Matsumura et al., 2003; Feng et al., 2008).** Formation of active oxygen as a result of silver nano interaction with enzymes may cause cell damage **(Morones et al., 2005)**. The suggested mechanisms of silver nanoparticles as antimicrobial agant were illustrated in **(Fig 12)**.

Table 2: Antimicrobial activities of different nanomaterials on the isolated fungal strains

Serial no	Fungal name	Clear zone (ϕmm)		
		Nanolime	Nanosilver (AgNPs)	Nanogel
1	Aspergillus terreus	12	14	0
2	Aspergillus sydowii	17	22	15
3	Paecilomyces variotii	17	20	0
4	Aspergillus clavatus	18	25	0
5	Fusarium culmorum	20	20	13
6	Aspergillus niger	22	24	15
7	Aspergillus humicola	20	12	0

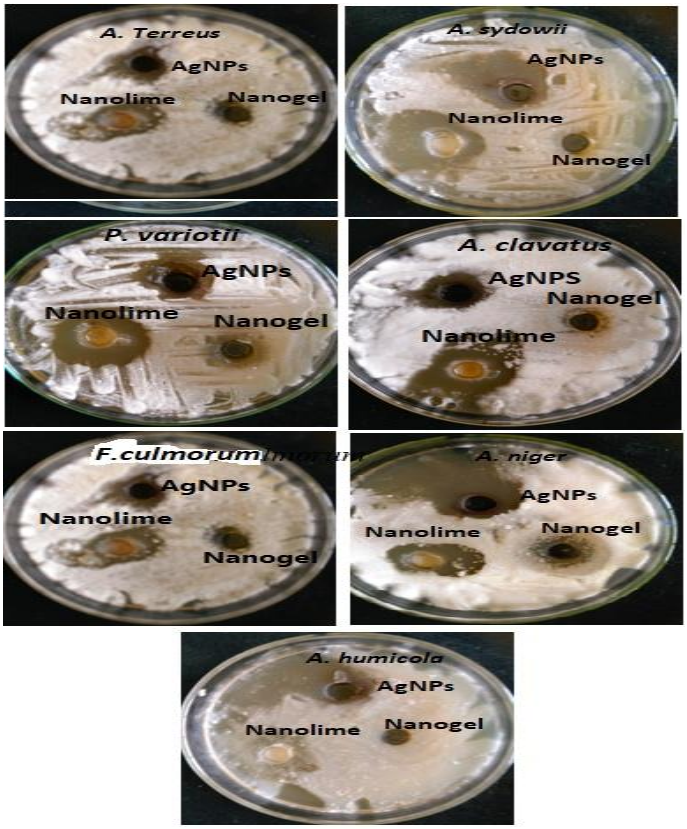


Figure 11: Antimicrobial activities of different nanomaterials on the isolated fungal strains.

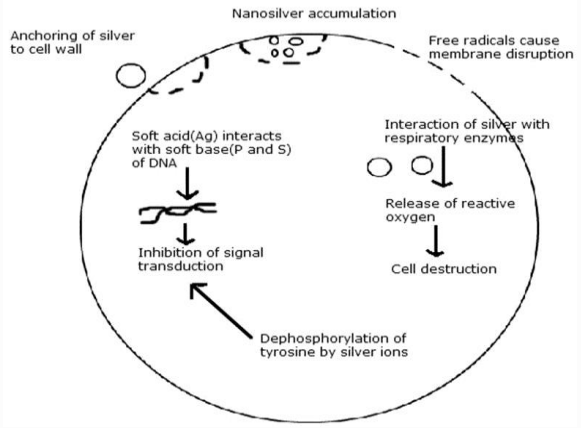


Figure12. Various modes of action of silver nanoparticles on bacteria (Prabhu and Poulose, 2012).

3-7 Effect of different pigments as antifungal agents

The effect of different pigments (Egyptian blue, Egyptian green, Azurite and Malachite) in its powder form or mixed with the binding media (animal glue) on the isolated fungal strain have been studied. (Fig 13) investigated the effect of the powder pigments on the different isolated fungi it has been found that both azurite, Egyptian blue and malachite exhibit antifungal against Aspergillus terreus, Fusarium oxysporum and Aspergillus niger and no antifungal activities have been found with the pigments (Egyptian green) against all other fungi. (Fig 14) showed the effect of the pigments mixed with animal glue on the isolated fungal swains. Realty (reuoled) that malachite, azurite and Egyptian blue exfoliated antifungal activities against Aspergillus niger and Fusarium while malachite only exhibited antifungal activity against Aspergillus terreus. The pigment did not show any antifungal activity against the other fungal. Egyptian green and did not show any and antifungal activity against all isolated fungi. Fungi were killed when exposed to higher concentrations of copper **(Borkow and Gabbay, 2005; Borkow, 2012).** This means that the responding of fungal strains was differed according to fungal strain and copper dose. Copper may damage the microorganisms through direct contact **(Grass, 2011; Bleichert et al., 2014; Santo et al., 2012)**. Other mechanisms include the damaging of envelop phospholipids, intercellular protein as well as damaging nucleic acid **(Ohsumi et al., 1988; Bleichert et al., 2014; Santo et al., 2012; Nan et al., 2008; Rifkind et al., 2001; Sagripanti et al., 1991; Kim et al., 2000; Karlstrom and Levine, 1991).**

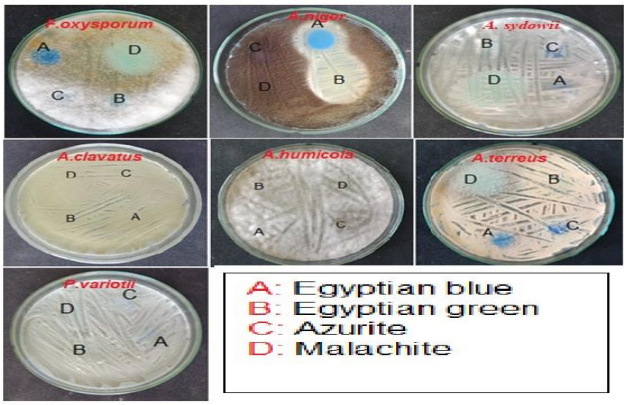


Figure 13: Effect of pigments in their powder form on different fungal strains isolated from Stelae in Egyptian Museum-Egypt.

Figure 14: Effect of pigments mixed with the binding media (animal glue) on different fungal strains isolated from Stelae in Egyptian Museum-Egypt.

3-8 Degradation of animal glue by isolated fungi

The ability of the fungi isolated from Stelae to degrade animal glue has been studied by cultivating them on Saboraud Dextrose broth supplemented by 2% animal glue for 7 days. The degradation has been evaluated by measuring the increase in growth represented as dry weight. (Fig 15\16) a&b showed the fungal growth in Erlenmeyer flasks and after centrifugation, respectively. Also, the dry weight (Table 3) was weighed in nitrocellulose membranes after drying at 80^{o}C for 24h and represented as g/100ml of culture filtrate. It has been noticed that Aspergillus humicola exhibited the highest dry weight (2.09629g/100ml) followed by Paecilomyces variotii (1.5655g/100ml), Aspergillus sydowii (1.3856g/100ml0, Aspergillus clavatus (1.3554g/100ml), Aspergillus terreus (1.1486g/100ml), Fusarium oxyspourm (1.1138g/100ml) and Aspergillus niger (0.4319 g/100ml). The proteolytic activity of mycelial free culture filtrates of fungal strains isolated from Stella has been also studied. It has been found that all the fungal isolated exhibited proteolytic activities but with different degrees. Results in Table – revealed that the proteolytic activites for all fungi followed the following order: Aspergillus clavatus (86.040U/ml) > Paecilomyces variotii (70.044U/ml) > Aspergillus Humicola (69.528U/ml) > Aspergillus terreus (60.948U/ml) > Aspergillus sydowii (57.108U/ml) > Aspergillus niger (46.092U/ml) > Fusarium oxyspourm (39.180U/ml). Protease activity (collagenase) from and the dry weight from the yeast 13II isolated from bee pollen have been studied **(Luiz et al., 2014)**.

Table 3: Total proteolytic activities of fungal strains isolated from Stella after cultivation on medium containing animal glue

No.	Fungus	Dry weight (g/100ml)	Total proteolytic activity (U/ml)
1	Aspergillus terreus	1.1486	60.948
2	Aspergillus sydowii	1.3856	57.108
3	Paecilomyces variotii	1.5655	70.044
4	Aspergillus clavatus	1.3554	86.040
5	Fusarium oxyspourm	1.1138	39.180
6	Aspergillus niger	0.4319	46.092
7	Aspergillus humicola	2.0962	69.528

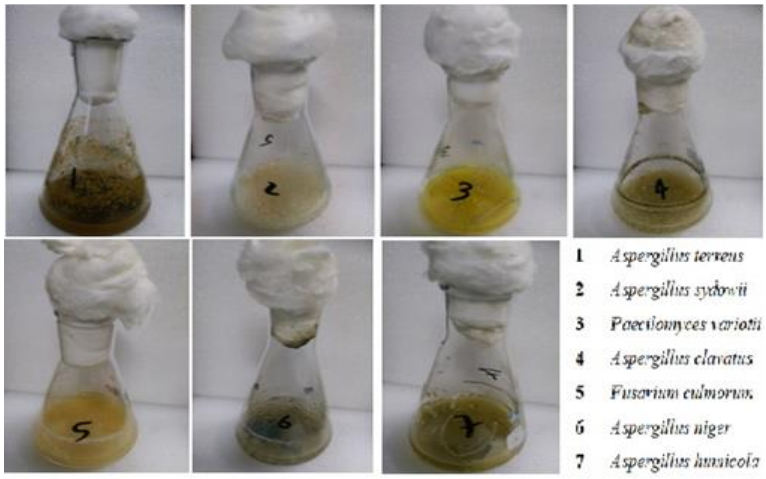


Figure 15: The growth of different fungi isolated from the Stelae on culture medium containing animal glue (Growth in flasks and b).

Figure 16: Shows the mycelia after centrifugation.

4- Conclusion

Different fungal strains were isolated from ancient mural painting that subjected to deterioration. These fungi were identified based on their morphological features. The effects of different pigments as well as their antifungal activities were investigated. To overcome the growth of fungi, different anaomaterials were prepared including; Nanogel, nanolime, and nanosiver. Nanosiver was found to be the best antifungal agent. The biodegradation of the binder (animal glue) has been also studied.

Acknowledgment

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