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The effect of nano-TiO_2 and plant extracts on microbial strains isolated from Theban ancient Egyptian royal tomb painting

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Mural paintings in ancient Egyptian tombs in West Thebes have been suffering from several deterioration factors and symptoms such as variations of temperatures and relative humidity, salts efflorescence and crypto-florescence, crackling and bio-deterioration effects, which assimilate in insects, algae, actinomycetes, etc. Other causing factors are bacteria and fungi, which accelerate mechanical weathering, chemical changes and aesthetic deterioration, like the penetration of mycelium below plaster layers, decomposition, disintegration, alterations and discoloration. These micro-organisms can excrete organic and inorganic acids, alkaline compounds, chelating, enzymes substances and pigments. Three fungi strains (*Fusarium oxysporum, Rhizopus stolonifer* and *Aspergillus flavus*) and two bacteria strains (*Staphylococcus warnei* and *Micrococcus luteus*) were isolated from the royal Theban tombs paintings (west Thebes, Luxor, Egypt). This work aimed to access the presence of microorganisms and their effect on mural paintings deterioration; it also studies their treatment methods, such as nanoparticles (TiO_2 NPs) and *Sesbania sesban* and *Ricinus communis* plant extract (PE). The applied doses of NPs and PE did not cause any observable alterations or color changes to pigments and binding media (arabic gum) used in the paintings. TiO_2 NPs 160 ppm and 100 mg of plant extracts were the efficient concentration level in eliminating microbial growth. The causes of the different efficacy of the treatments are observed, as well as the potential risks of recolonization by viable cells left behind after treatment.

Key words: Bio-deterioration, Plant extracts, Wall painting, West Thebes tombs-Nanoparticles.

INTRODUCTION

Bio-deterioration can be defined as “any undesirable change in a material brought about by the vital activities of organisms” (Allsopp, 2011). Bacteria, actinomycetes and fungi are constantly causing problems in the conservation of mural paintings tombs because of their biodeteriorative potential. The Thebes town is one of the largest and famous archaeological sites in the world. It lies about 900 km South of Cairo on the banks of the

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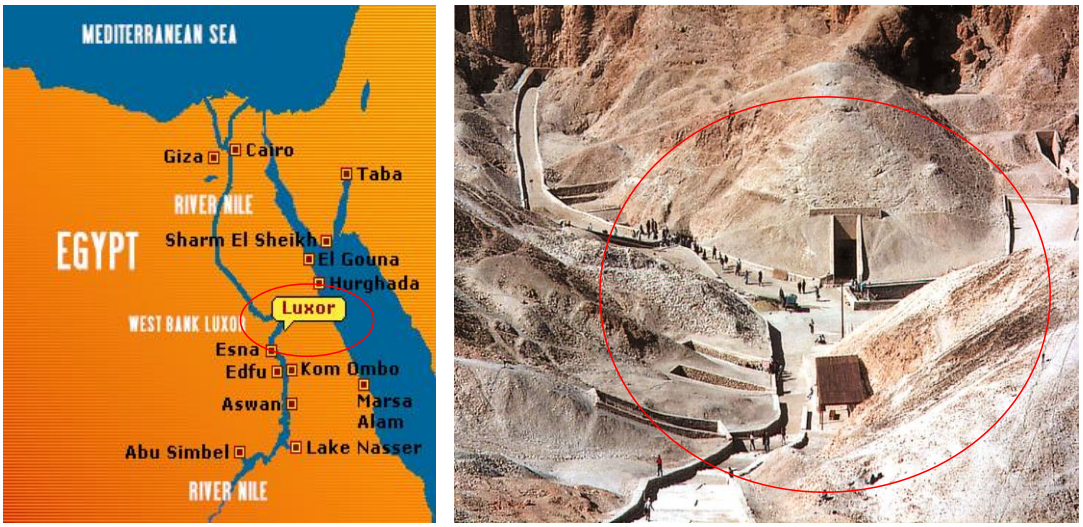

Figure 1. Plan of Luxor and west bank Luxor.

River Nile. The tradition of wall painting is very old, dating back to prehistoric times (Garg et al., 2010). Royal tombs in Thebes (west Bank, Modern Luxor) are as considered one of the most important archaeological features in Egypt and the world over. That is, where this archaeological site is the most important period of Egyptian history, tombs were constructed for the Pharaohs and powerful nobles of the New Kingdom (the Eighteenth to the Twentieth Dynasties of Ancient Egypt). Wall painting generally classified as tempera or fresco depending on the technique of execution, possesses a layered structure consisting of support, ground and paint layer (Garg et al., 2010). Wall painting supports the growth of microorganism commonly involved in bio-deterioration, contributing to the destruction of paint; their deterioration constitutes a loss affecting a significant part of the world's cultural heritage (Pepe et al., 2010). The growth of fungi on wall paintings manifested most commonly by discoloration or deterioration of the surfaces and microbial popullation causes discoloration of pigments, physical decay when fungal hyphae grow either on or below the surfaces, cause chemical decay of paintings through their metabolites and enzyme production like gluconic, citric, oxalic, malic etc (Pepe et al., 2010; Hideo, 2000; Dornieden et al., 2000; Garg et al., 2010). Biodegradation and bio-deterioration is a serious risk to cultural heritage, which needs the application of effective and fast methods in order to identify the microorganisms involved in this process and to assess their biodegradation and bio-deterioration ability (Pangallo et al., 2009). Their biological attack occurs at favourable temperature and relative humidity conditions for the development of microorganisms and spores present on the substratum. Each colonizer agent has different ways to compromise the structure in function of the substrates (Nugari et al., 1993; Borrego et al., 2010). Nanoparticles have antimicrobial effect such as silver nano particles, copper nanoparticles, zinc oxide nanoparticles and titanium oxide nano particles etc (Stoimenov, 2002; Ip et al., 2006; Ren et al., 2009; Zhang et al., 2007). In this study we used two methods to inhibit the isolated microbial from the surface of wall painting: the first involves using titanium dioxide nanoparticles with a different level of concentration and the second method entails using two tradition plant extracts- Sesbania (*Sesbania sesban*) and Ricinus (*Ricinus communis*).

MATERIALS AND METHODS

Samples and site description

Samples for the study were taken from different parts of the royal tombs such as Tausert and Setnkht, Seti I., Ramsis v., Ramsis vi. (Figure 1) according to scientific and not destructive methods. The biological samples were taken by sterile cotton swap and incubated for 7 days; the isolated fungi were identified according to morphological and spore structures (Barnett et al., 1972; Sun et al., 1978).

Three isolates of fungi and two of bacteria were collected from different tombs; 900 km Southeast of Cairo, during March 2014 by using the sterile cotton swap method. Samples were taken from paintings of yellow, red, black, blue color and stone surfaces in investigated tombs (Figure 2). They were inoculated on nutrient agar and potato dextrose agar, and incubated for a period of 5 days at 30°C.

Preparation of plant extracts

The fresh plant leaves were brought to the laboratory and washed under running tap water; they were air dried and then homogenized to fine powder and stored in airtight bottles. The dried powder of *S. sesban* and *R. communis* was successively mixed with methanol for

Biodeterioration of wall painting (Seti ı tomb)

Detailed view show fungal degradation (discoloration, detachment of plaster and pigments

Figure 2. Biodeterioration of wall tomb painting.

Table 1. Microbial inhibition zone (mm) of plant extracts concentration (mg/ml).

Test organism	Sesbania extract concentration (mg/ml)				Ricinus extracts concentration (mg/ml)			
	20 mg	40 mg	80 mg	100 mg	20 mg	40 mg	80 mg	100 mg
F. oxysporum	9	12	13	13	8	10	13	13
R. stolonifer	10	10	12	13	7	10	11	12
A. flavus	8	9	11	12	9	12	12	13
S. warnei	8	9	12	14	8	12	12	14
M. luteus	10	11	12	15	10	11	12	13

72 h. The extracts were dried under reduced pressure using rotator evaporator to get the crude. A dark green semi-solid mass was obtained. It was stored below 4ºC until further used. The alcohol extract was prepared by adding 10 g of crushed leaves of sesbania or ricinus in 50 ml of methanol separately. It was allowed to stand for 24 h after which it was filtered using a Whatman No. 1 filter paper. The filtrate was directly used as crude extract with 100% concentration. Further dilutions were made by adding appropriate amount of methanol.

Microbial culture and media

The microbial cultures were prepared in microbiology agriculture lab and used in the present study. The bacteria rejuvenated in nutrient broth at 37°C for 18 h and then stocked at 4°C in nutrient agar (Khante et al., 2008). Subcultures were prepared from the stock for bioassay. The inoculum size of the bacterial culture was standardized according to the National committee for Clinical Laboratory Standards guideline. The bacterial culture was inoculated into sterile nutrient broth and incubated at 37°C. The fungi medium is Potato Dextrose Agar PDA (200 g potato infusion, 20 g agar + 20 g Dextrose+1 L distilled water) and the bacterial medium is nutrient agar NA (0.5% peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% NaCl, 1 L distilled water).

Preparation of disc for antimicrobial activities

The sterile blotting paper disc (5 mm) was soaked in the diluted extract in different concentrations (20, 40, 80 and 100%). The prepared disc was dried in controlled temperature to remove excess of solvent. The modified paper disc diffusion (Delignette-Muller and Flandrois, 1994) was employed to determine the antimicrobial activity of aqueous extract of the herbal preparations. Inoculum was spread over the agar plate using a sterile cotton swab in order to obtain uniform microbial growth. Then the prepared antimicrobial discs were kept over the lawn and pressed slightly along with control. Streptomycin 10 µg/disc was used as positive control. The plates were incubated for 48 h at 37°C. The antimicrobial activity was evaluated and diameter of inhibition zones was measured. Experiment was carried out in triplicate and the averages diameter of zone of inhibition was recorded. The antibacterial activity was classified as highly active (>10 mm), mildly active (7-10 mm) and slightly active (6-7 mm); and less than 6 mm was taken as inactive (Chandra et al., 2011).

RESULTS AND DISCUSSION

Effect of plant extracts on microbial strains

The antimicrobial activity of methanol extracts of *S. sesban* stem portion was studied against *Staphylococcus warnei* and *Micrococcus luteus* by zone of inhibition method. The dried leaves of *S. grandiflora* are used in some countries as tea which is considered to have antibiotic properties (Gupta et al., 2008). The zone of Inhibition study results are depicted in Table 1. The result indicates that after 48 h methanol extracts (100 mg/ml) showed zone of inhibition of 15 mm against *M. luteus,* and 14 mm against *S. warnei*. The fungal group zone of

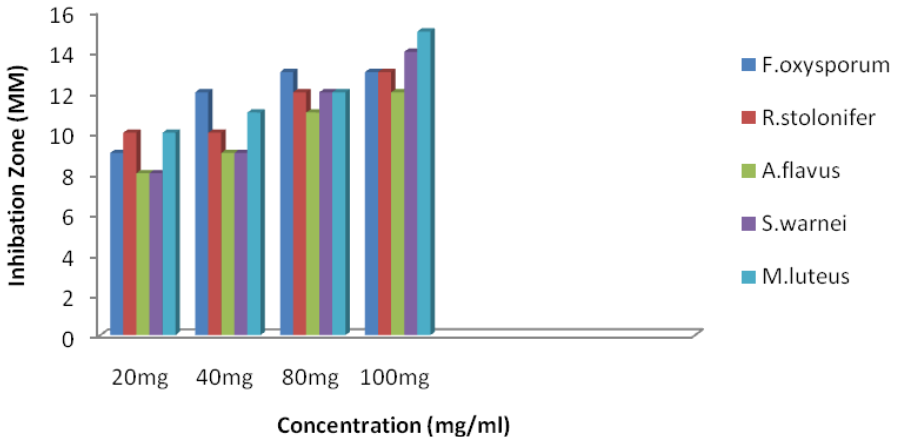

Figure 3. Effect of different sesbania concentration on microbial inhibition zone.

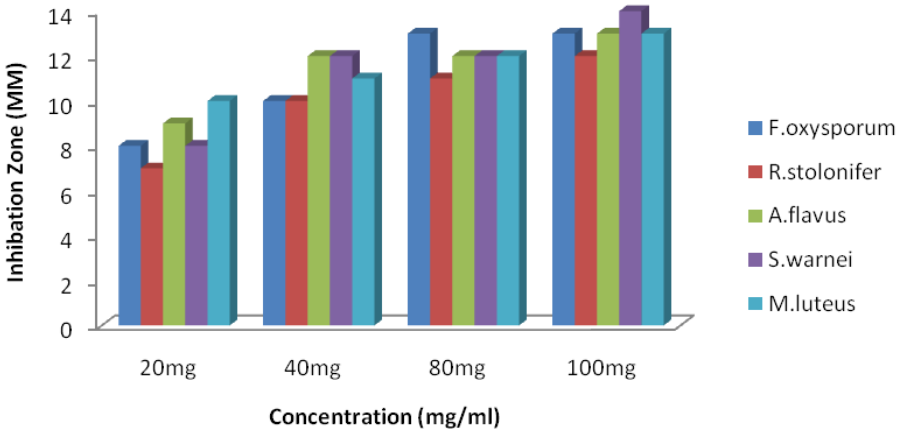

Figure 4. Effect of different Ricinus concentration on microbial inhibition zone.

inhibition (100 mg/ml), after 72 h, was 12.3 mm against *Fusarium oxysporum;* 12.1 mm against *Rhizopus stolonifer* and 12.6 mm against *Aspergillus flavus.* The graphical representation of zone of inhibition vs different concentration of plant extracts after different time intervals (48 h for bacteria and 72 h for fungi) are shown in Figures 3 and 4 respectively.

According to Sandeep et al. (2014), cloroform and methanol extracts of stem of *S. sesban* (25 mg/ml) equally inhibit *P. aeruginosa* after 48 h. Both chloroform and methanol extracts at a concentration of 100 mg/ml were more effective against *B. subtilis* than other two bacterial strains after 48 h. No antibacterial activity was observed for pet ether ethanol and water extracts in a range of 25 to 100 mg/ml. The methanol leaf extract of *R. communis* exhibited maximum antimicrobial activity, and water extract showed minimum activity against four bacteria (*S. aureus*, *B. subtilis*, *P. aeruginosa* and *K. pneumonia)* and two fungal strains (*A. fumigatus* and *A. flavus).* These results are in agreement with the previous work which showed that in plants most of the compounds having antimicrobial potential are soluble in methanol (Chandrasekaran et al., 2004), and low activity of water extract is also reported by Ashafa et al. (2008).

R. communis showed good activity against *M. luteus, S. warnei, F. oxysporum, R. stolonifer* and *A. flavus*. The antimicrobial assay revealed that the methanol extracts of leaves of *R. communis* possess good zone of inhibition, whereas alcohol extract has antimicrobial activity only in higher concentration (Table 1). Significant susceptibility was recorded by most of the organisms tested with methanol extract of leaves of *R. communis*, which showed a comparatively reduced susceptibility pattern (Figure 5).

The susceptibility pattern exhibited by the tested organisms to these extracts could be exploited for probably medicinal purposes in chemotherapy among humans. With the current spread of antibiotic resistance almost at geometric scale (Olayinka et al., 2004), proper attention should be given to such plants to reap the

Figure 5. Inhibition zone of antibacterial activity of TiO_2 nanoparticles and plant extracts at different.

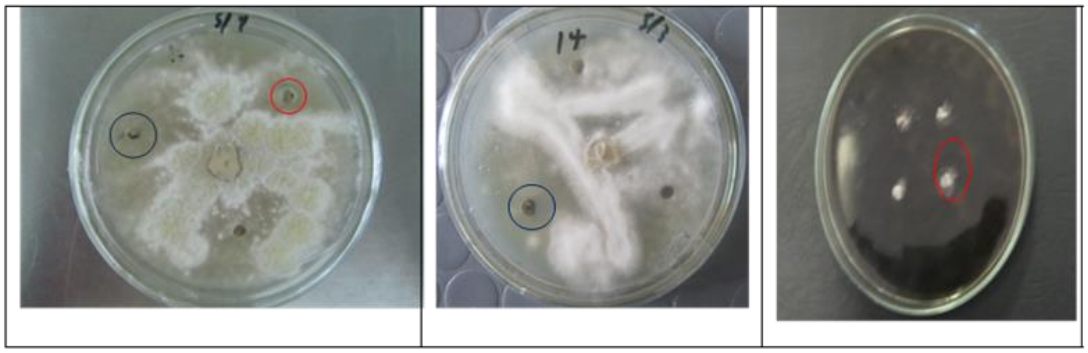

Figure 6. Inhibition zone of antifungal activity of TiO_2 nanoparticles and plant extracts at different concentrations.

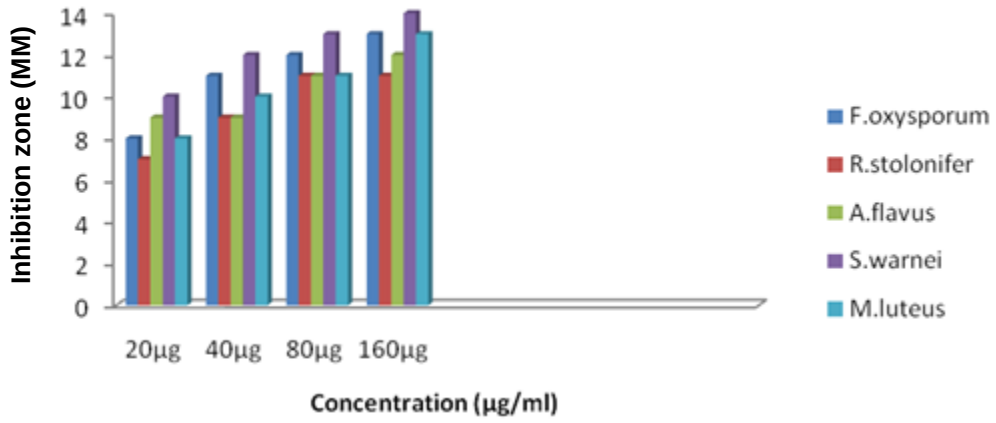

Figure 7. Effect of different nano TiO_2 concentration on microbial inhibition zone.

potential antimicrobial benefits inherent in them. However, actual antimicrobial ingredients need to be extracted and identified; also its tolerable levels in the human body as well as any toxic effects on human and animal tissues need to be investigated accordingly.

Effect of Nano-TiO_2 on microbial growth

The effect of chemical synthesized TiO_2 nanoparticles on the growth of microbial strains is shown in Figures 6 and 7. The antimicrobial activity is probably derived, through

Table 2. Microbial inhibition zone (mm) of Nano TiO_2 concentration (µg/ml).

Test organisms	20 µg	40 µg	80 µg	160 µg
F. oxysporum	8	11	12	13
R. stolonifer	7	9	11	11
A. flavus	9	9	11	12
S. warnei	8	12	13	14
M. luteus	8	10	11	13

the electrostatic attraction between negatively charged cell membrane of microorganism and positively charged nanoparticles (Hamouda et al., 2001; Dibrov et al., 2002; Dragieva et al., 1999), interaction of metal ions including titanium with microbes (Amro et al., 2000) and orientation of TiO_2 (Wang et al., 2007).

Chemical TiO_2 treatments had significant inhibitory effect on the growth of microbes during 24 and 72 h of incubation. The growth of the medium was investigated as the number of microbes after contact with nano-particles. The growth inhibition of the microbe by both treatments recorded as a function of time suggested significant differences in antimicrobial activity of the nanoparticles (Table 2).

Chemically synthesized TiO_2 NPs has a stronger inhibitory effect on microorganisms. TiO_2 NPs at a concentration 20, 40, 80 and 160 inhibited growth of bacterial and fungal strains, whereas the effect was much less at lower concentration (Kon and Rai, 2013). The efficacy of antimicrobial in terms of zones of inhibition (mm) was measured against five different fungal and bacterial strains (Table 2). The decreasing order of the average antimicrobial activity of NPs against fungal group was observed to be *F. oxysorum* > *A.flavus* > *R. stolonifer*. The average antimicrobial activity of the various NPs concentrations against bacterial group and fungal group inhibition zone ranged from 8–14 and 8-13 mm, respectively. Increasing concentration of TiO_2 nanoparticle decreases the growth of microbes, and the concentration at which growth stopped altogether was high with TiO_2 chemically synthesized nanoparticle. According to Adams et al. (2006), nanoparticles inhibited growth of gram-positive by 90% but gram-negative was much more resistant.

The antimicrobial ability of nano-TiO_2 might be referred to their small size which is 250 times smaller than a bacterium. This makes them easier to adhere with the cell wall of the microorganisms causing its destruction and death of the cell. Also, metal nano-particles are harmful to bacteria and fungi (Chwalibog et al., 2010). Nano-TiO_2 stimulates biofilm production and aggregate within this bio-film. They bind closely to the surface of microorganisms causing visible damage to the cells, and demonstrating good self-assembling ability. Nano-TiO_2 possesses well-developed surface chemistry, chemical stability which makes them easier to interact with the microorganisms (Nirmala and Pandian, 2007). The particles interact with the building elements of the outer membrane and might cause structural changes, degradation and finally cell death. The effectiveness of TiO_2 can be explained on the basis of the oxygen species released on the surface of TiO_2, which cause fatal damage to microorganisms (Sunada et al., 1998). The generation of highly reactive species such as OH^-, H_2O_2 andO_{2-} is explained as follows. Since TiO_2 with defects can be activated by both UV and visible light, electron-hole pairs (e^-h^+) can be created. The holes split H_2O molecules (from the suspension of TiO_2) into $OH^-$and H^+. Dissolved oxygen molecules are transformed to superoxide radical anions ($\bullet O^{-2}$), which in turn react with H+ to generate ($HO_2\bullet$) radicals, which upon subsequent collision with electrons produce hydrogen peroxide anions (HO^{2-}). They then react with hydrogen ions to produce molecules of H_2O_2. The generated H_2O_2 can penetrate the cell membrane and kill the bacteria (Fang et al., 2006). Since the hydroxyl radicals and superoxide are negatively charged particles, they cannot penetrate into the cell membrane and must remain in direct contact with the outer surface of the bacteria cell of the bacteria; however, H_2O_2 can penetrate into the cell (Blake et al., 1999). The mechanisms of TiO_2 nanoparticles depend on the bactericidal effect which is a result of mechanical interactions on cell walls and/or membranes of microorganisms in the presence of photocatalysis.

Conclusion

In this work we have reported the evidence of toxic effects of Titanium dioxide nanoparticles (TiO_2 NPs) and plant extracts on different pollution microorganisms. The antimicrobial activity of ethanol extracts was evaluated by disc diffusion plate method. All the extracts were evaluated in concentration range from 20 to 100 mg/ml. Wall painting in the royal tombs (Tausert - Setnkht and Seti I) suffers from microbial enzymes degradation and needs urgent treatment. The lab test was performed by using various concentrations of TiO_2 NPs; 160 ppm was the efficient concentration level in eliminating microbial growth. TiO_2 NPs and plant extracts did not cause any observable alterations or color changes to pigments and binding medium (Arabic gum) used in wall paintings. The order of bacterial strains inhibition of methanol extract after 48 h is as follows: *S.warnei* >*M. luteus*> *F.oxysporium*> *A. flavus* > *R. stolonifer*. So, it is concluded that Sesbania and Ricinus extracts possess good antibacterial activity. The antibacterial activity may be due to the presence of flavonoids and phenolic compounds.

Conflict of interests

The authors did not declare any conflict of interest.

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