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Effect of Thymol against Fungi Deteriorating Mural Paintings at Tell Basta Tombs, Lower Egypt

Akmal A. Sakr[1, 2], Mohamed F. Ghaly [2], Gamal E. Helal [2], Mahmoud E.F. Abdel Haliem[2]

[1]*Conservation Department, National Museum of Egyptian Civilization (NMEC), Cairo, Egypt*

[2]*Botany Department, Faculty of Science, Zagazig University, Zagazig, Egypt*

[3]*Conservation Department, Faculty of Archaeology, Cairo University, Cairo, Egypt*

Corresponding Author:** ***Akmal A. Sakr, *Conservation Department, National Museum of Egyptian Civilization (NMEC), Cairo, Egypt*

Abstract: *Twenty five fungal isolates were collected from mural paintings, air and stone surfaces at Tell Basta tombs and were attributed to Aspergillus versicolor, A. japonicus, A. restrictus, A. terrus, A. fumigatus and A. nidulans, Fusarium oxysporum and Penicillium sp. Moreover, A. japonicus was the, most present in the total fungal samples.*

The isolated fungi showed to be involved in fading of azurite blue and red hematite pigments due to the bioaccumulation of copper (Cu) and iron (Fe) ions, respectively, on the fungal cell wall. Fe ions enhanced the biomass, but Cu ions enhanced melanin production.

Thymol was used with different concentrations (10, 20, 30, 50, 100 µg/ml) to inhibit the fungal growth and the concentration 100 µg/ml was the most effective. Isolated fungi varied in their resistance profile to the tested thymol concentrations since Penicillium sp. was the most resistant, but A. japonicus was the most sensitive.

Thymol caused yellowing of the treated surfaces, so fumigation method was recommended. Furthermore, fumigation reduced the microbial colonization of paintings significantly. On the other hand, thymol caused color change neither for the tested pigments nor for the appearance of stone surfaces.

Keywords: *Biodegradation, Fumigation, Mural Paintings, Tell Basta, Thymol, Discoloration, Upper Egypt*

1. INTRODUCTION

Mural paintings within Tell Basta (Bubastis) tombs are often subjected to fungal deterioration, since fungi play an important role in biodeterioration of mural paintings. Fungi cause chromatic alteration through forming irreversible stains resulted in secreting extracellular in nature of melanin and carotenoid pigments (Sterflinger et al. 1999), even after the fungus is dead, the pigmented cell walls remain on the surface of the paintings and stone surfaces and these strains are resistant to chemical and enzymatic degradation (Kiyuna et al. 2011). The structural damage is caused by fungal metabolites of acids that are extremely erosive (Sterflinger, 2010) and these acids cause chromatic alterations of paintings (Ravikumar et al. 2012). Furthermore, produced enzymes could decompose the binding media of the mural paintings, such as egg yolk, animal glue and arabic gum into free amino acids and mono sugars that could be used as carbon source for fungal growth and colonization (Milanesi et al. 2006).

Fungal deterioration of mural paintings in ancient Egyptian tombs was put onto the evidence, since mural paintings in the tomb of the King Tutankhamen (18th Dynasty) were stained with brown-black spots, that resulted in the growth and colonization of *Aspergillus penicilloides* (Arai 2004; Vasanthakumar et al. 2013). Furthermore, *Cladosporium* sp. disfigured mural paintings in the tomb of the King Amenophis III (18th Dynasty) by forming black stains (Arai 2004). Also *Penicillium*, *Aspergillus*, *Alternaria* and *Cladosporium hebarum* formed black stains on mural paintings within the tomb of Petosiris that dated back to the Ptolemaic period (El-Deeb et al. 2000). Moreover, *Cladosporium* showed to be involved in disfiguration of stone surfaces in the tomb of the Queen Nefertari (18th Dynasty) with black spots (Preusser 1987). In addition, *Cladosporium cladosporoides*, *Alternaria alternata* and *Aspergillus niger* caused discoloration of mural paintings in the Temple of Abydos (19th Dynasty) with dark brown stains (El Sharouny et al. 2000b).

Due to the aesthetical and structural damages that fungi are causing to mural paintings and stone surfaces, their growth should be controlled.

Biocontrol appears to be a reliable alternative to chemical fungicides, because the applied fungicides are highly toxic and impose environmental hazards for both the treated objected and conservators, so the new trends used environmentally safe methods of plant extracts and essential oils such as thyme and its derivatives to control fungi colonizing cultural heritage objects. These substances have high potent antimicrobial activity and low toxicity for human and environment (Unger et al. 2001; Haines and Kohler 1986).

Thymol is the most common constituent of essential oils derived from *Thymus* and *Origanum* plants, and confers antimicrobial properties to these oils (Liang et al. 2007), that may explain its plant origin as a chemical defense mechanism against phytopathogenic microorganisms (Numpaque et al., 2011). This antimicrobial activity is attributed to the phenolic compounds that have a wide spectrum of antimicrobial activity against fungi and bacteria (Hu et al. 2008; Rota et al. 2008; Soumya et al. 2011).

Because of its efficacy, it is often used as preservative agent for organic cultural heritage objects such as library and other archival materials made of paper (Veilkova et al. 2011), leather and parchment (Sirvaityte et al. 2012).

Because thymol is only fungistatic, and no fungicidal effect was recorded, so it could be used in combination with other substances such as antibiotics (Ahmad et al. 2010) and mercuric chloride (Sadurska and Kowalik, 1968) due to the synergistic effect of thymol and antibiotics or mercuric chloride.

To date, several problems have been encountered in application of thymol with diffusion method for inhibiting fungi colonizing cultural heritage objects, such as turning yellow at exposure to sun light or UV radiation for a long period. This yellowing phenomenon was attributed to the oxidation of phenol groups and hydroxyl group that transformed into carbonyl group and thymoquinone or dithymoquinone with yellow color strongly absorbed in FT/IR spectra (Daniels and Boyd 1986). For this reason, thymol was used exclusively since 1983 as preservative agent for archives in the United Kingdom in fumigation within closed boxes containing thymol, called fumigation chambers (Craig 1986).

The fumigation method is more effective than the contact one in controlling biodeteriorated cultural heritage objects (Daniels and Boyd 1986; Collis, 1970). This may be attributed to the matter of fact that fumigation provides a continual presence of thymol vapors within the exhibition cases that remain for weeks or even months after fumigation, thus reducing the numbers of viable spores able to recolonize these objects in the future. Furthermore, fumigation leaves no residues on organic cultural heritage objects such as parchment or paper (Haines and Kohler1986; Strassberg1978).

Efficacy of thymol fumigation should depend on the application conditions, such as type of fungi, concentration of thymol and relative humidity of the surrounding environment since it was reported that thymol fumigation is more effective if the objects are dried or in an environment with low humidity (Unger et al. 2001).

This mode of action of thymol vapors on the treated fungi may be attributed to disruption of membrane integrity in fungi (Liang et al. 2007) and this structural distortion would cause deterioration of the membrane and would increase membrane permeability (Altiok et al. 2010).

Finally, thymol fumigation could be used as preventive agent after the treatment of deteriorated cultural heritage using gamma irradiation due to short term effect of gamma irradiation (Abdel Haliem et al., 2013a)

The objective of this work was to isolate and characterize fungi colonizing mural paintings in the tombs at Tell Basta area, and to examine the resistance profiles of isolated fungi to different concentrations of thymol. Finally, the effect of thymol on different pigments is described herein.

2. Materials and Methods

2.1. Sampling Location

Twenty five samples of fungi were collected from paintings, stone surface, and air within five tombs (Ankh h3 f, Ihy, Ist, Ankh m b3st and unknown tomb) at Tell Basta (Bubastis in the Greec Roman

Period), East of Delta, Sharkyia Governorate that were discovered in 1980s-1990s (Figure.1), sampling was through June-July 2010.

Figure1a.*The location of sampling site*

Figure1b. *Location samples from Tell Basta tombs*

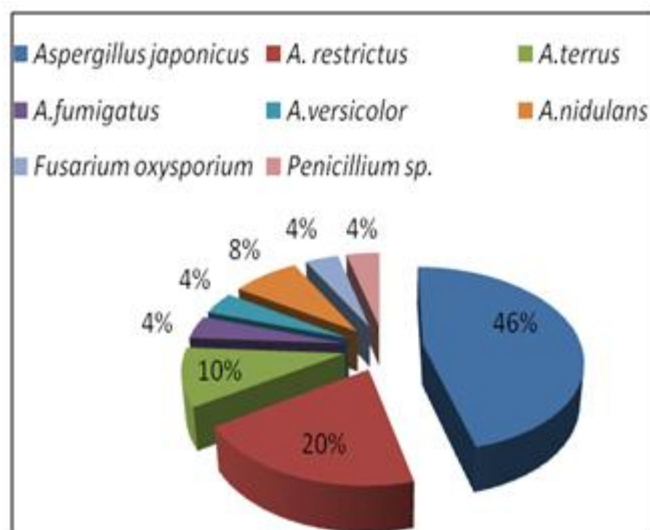

Figure2. *Percentage of fungal isolates*

After sampling, to extract microorganisms, sterile cotton swabs were aseptically suspended in 1 ml saline (0.9 % NaCl), amended with 0.1 ml Tween 80) (Descheemaeker and Swings, 1995) and vortexed for 10 min using a programmable rotator-mixer (model Mutli RS-60). Thereafter an aliquot of 0.1 ml was cultured onto Dox-Czapek agar medium plates (g/l) (sucrose 30, K_2HPO_4 1, $NaNO_3$ 3, $MgSO_4$. 7 H_2O 0.5, KCl 0.5, $FeSO_4$. $5H_2O$ 0.01, agar 20/L distilled water) supplemented with antibacterial chloramphenicol (0.05 g/l) to inhibit the rapid growth of bacteria, plates were incubated for 5 days at 25°C, where micro fungi appeared (Šimonovicova et al. 2004). Air samples were collected from the air within the investigated tombs according to Abdulla et al. (2008) using automated air sampler (Microbio Air Sampler MB2, Parrett LTD, UK) where fixed volume of air samples were collected over 1 min, directly onto Dox-Czapek agar medium plates, incubated for 5 days at 25°C.

2.2. Isolation and Identification of Fungi

Micro fungi were identified microscopically and morphologically according to the identification keys of Booth (1977); Raper and Fennell (1977); Raper et al. (1968), and this classification was confirmed by analysis of 18S rDNA sequence of these isolates.

2.3. Effect of Copper and Iron Based Pigments on the Isolated Fungi

To investigate the effect of iron based and copper based pigments on the growth of the isolated fungi, fungal isolates were cultured on broth Dox-Czapek medium, since 250 ml Erlenmeyer flasks were used, each one contained 20 ml, supplemented with 0.01% $CuSO_4$.7H2O and anhydrous ferrous sulphate ($FeSO_4$) respectively, incubated for 7 days at 28°C. Cu and Fe ratio in the biomass were estimated according to Humar et al. (2004). The fungal biomass was washed with distilled water and burnt in an oven at 150°C for 12 h to get rid of organic matters. 1g of burnt biomass was dissolved in 2 ml HOCl + 2 ml HNO_3 + 20 ml distilled water and left over night. Ratio of Cu and Fe ions in both treated and control samples cultured onto Dox plates free Cu and Fe ions were estimated in ppm/g using atomic absorption (Unica 969 Atomic Absorption Spectroscopy, Veternary Medicine, Zagazig University).

2.4. Acidolytic Activity of Isolated Fungi

To examine the acidolytic activity of isolated fungi. Erlenmeyer (250-mL) flasks were used. Each flask contained 50 mL of the broth Dox-Czapek medium, fungal isolates were inoculated and incubated at 28 C for 72 h. After incubation period, 0.1 ml of medium was injected in HPLC National Research Centre, Dokky, Giza. Standard organic acids were used.

2.5. The Chemical Composition of Thymol

To have better understanding of mortality mechanism of thymol on isolated fungi, its chemical composition must be investigated, this was carried out using:

2.5.1. Mass Spectra

Thymol sample was identified using Mass spectra (JEOL, JMS-AX500 Mass spectra, National Research Centre, Dokky, Cairo).

2.5.2. H NMR Spectra

Thymol was analyzed with NMR (JEOL-ECA 500 Mega Heritz, National Research Centre, Dokky, Cairo).

2.5.3. FT/IR Spectra

The chemical structure of thymol was analyzed by FT/IR spectroscopy (JASCo. FT-IR 6100, National Research Centre, Dokky, Cairo) according to Derrick et al. (1999).

2.6. Effect of Thymol on the Isolated Fungi

Thymol, mainly in its crystalline form was provided by Sigma Chemical Co., Cairo. Thymol has poor solubility in water, so it was used in dimethyl sulphoxide (DMSO) with different concentrations (10, 20, 30, 50, 100 µg/ml). Filter paper discs saturated with the solution were used according to Sakr et al. (2012).

On the other hand, the fumigation cabinet containing thymol as a fumigant was used according to Rakotonirainy and Lavedrine (2005), where models of colonized objects were placed on a small mesh of polypropylene within a glass jar and the colonized surface was in the face of thymol solution. Thymol solution was placed in a Petri dish (d 7 cm) and a container with 250 ml with salt solution of ammonium nitrate and magnesium sulphate within the fumigation chamber to regulate the relative humidity (RH) level at 50% and 80% respectively. After exposure of the colonized models to thymol vapors (3-14 days), swabs were taken from different zones of these models and cultured onto fresh Dox-Czapek plates to detect viability of fungal spores. Survival of spores was normalized against control samples of non fumigated fungi.

To study the mode of action of thymol on the isolated fungi, control and treated samples of *A. japonicus,* the most present fungus, were examined using scanning electron microscope (JOLE. SEM 6300, National Research Centre, Cairo) according to Milanesi et al., (2006).

2.7. Schematic of Fumigation Chamber

The traditional fumigation chamber cabinet was modified whereas this chamber was supplemented with data logger to monitor relative humidity and temperature and with small fan to make air circulation so thymol vapors contact with fungal spores for longer period.

2.8. Effect of UV and Sun Light on Thymol

To explain the yellowing phenomenon of thymol, a solution of thymol in dimethyl sulfoxide (DMSO) was exposed to both sun light and UV lamps (TUV 40w) for seven days. Thymol traces were analyzed by FT/IR spectroscopy (JASCo. FT/IR 6100, National Research Centre, Dokky, Cairo), IR patterns were compared against the control samples of thymol.

2.9. Effect of Thymol on Different Pigments

To investigate the effect of thymol on pigments used in mural paintings, different pigments such as hematite, limonite, azurite, malachite, carbon black, red lead, cinnabar and lead carbonate were tested using different concentrations of thymol. The potential changes in the composition of pigments due to effect of thymol were determined by FT/IR spectroscopy (JASCo. FT/IR 6100, National Research Centre, Dokky, Cairo).

3. Results

3.1. Identification of Fungal Strains

The identification based on culture-dependent techniques showed that twenty five fungal strains isolated from Tell Basta tombs (from air, paintings and stone surfaces) were belonging to *Aspergillus japonicus* (46%), *A. restrictus* (20%), *A. terrus* (10%), *A. fumigates* (4%), *A. versicolor* (4%), *A.*

nidulans (8%), *Fusarium oxysporum* (4%) and *Penicillium* sp.(4%) (Figure.3a). On the other hand, it was found that 30 % of fungal isolates were from air, 27 % from limestone surfaces and 43 % from paintings, indicating selectivity of fungi in biodeterioration (Table 1).

Biochemical identification was confirmed using molecular methods, and 18S rDNA sequencing pointed out that isolated fungi are belonging to Aspergilli, Penicilli and Fusarium genera and their accession numbers are illustrated in Table 2.

Figure3. *Producing pinky pigment by F. oxysporum*

Table1. *Location of fungal isolates*

Fungal isolates	Location
A. versicolor (Vuill.)	Limestone, tomb of Ankh h3 f
A. japonicus Saito	Air, tomb of Ankh h3 f
Fusariumoxysporum Schlech	Limestone unknown tomb
A. restrictus Smith	Limestone unknown tomb
A. japonicus Saito	Limestone, tomb of Ankh m b3st
A. restrictus Smith	Air, tomb of Ankh m b3st
A. restrictus Smith	Air, tomb of Ankh m b3st
A. terreus Thom	Air, tomb of Ankh h3 f
A. restrictus Smith	Air, tomb of Ankh h3 f
A. japonicus Saito	Air, tomb of Ihy
A. japonicus Saito	Black color, tomb of Ihy
A. terreus Thom	Air, tomb of Ist
A. japonicus Saito	Limestone, tomb of Ist
A. japonicas	Red color, tomb of Ankh m b3st
A. nidulans (Eidam) Wint	Red color, tomb Ankh m b3st
A. japonicus Saito	Yellow color, tomb of Ankh m b3st
A.japonicus Saito	Limestone, tomb of Ankh h3 f
A.fumigatus Fresenius	Black color, tomb of Ankh h3 f
A.terreus Thom	Red color, tomb of Ihy
A.japonicus Saito	Blue color, tomb of Ihy
Penicillium sp.	39GwAQ7PeH7fJTFa4DXguurfn7GULq2pT
Penicillium sp.	Black color, tomb of Ihy
A. versicolor (Vuill.)	Red color, tomb of Ankh m b3st
A. japonicus Saito	Air, tomb of Ihy

Table2. *Phylogenetic affiliation of inoculated strains (homology of 18S rDNA and similarity in comparison with NCBI data)*

Sampling location	Affiliation	Similarity to NCBI 16S rDNA	NCBI accession number sequence
Azurite blue, tomb of Ihi, Tell Basta	*A. japonicus* Saito 99 %	BankIt	
Yellow color,Southern Wall of the tomb of Ankh h3 f Tell Basta	99%	BankIt1507642	JQ625332
Blue color, ceiling of the tomb of Oserkon II, Tanis	99%	BankIt1507650	JQ625337
Black color, tomb of Ankh h. f.,Tell Basta	100%	BankIt1507649	JQ625336
Red color, tomb of Ankh m b3st,Tell Basta	99%	BankIt1507648	JQ625335
Limestone, tomb of Oserkon II, Tanis	98%	BankIt1507647	JQ625334
North wall, tomb of Ist, Tell Basta	98%	BankIt1507149	JQ625330
Yellow color,southern wall of the tomb of Ankh h3 Tell Basta	99%	BankIt1507645	JQ625333

[a]*Alternative affiliation is S. pyridomyceticus.*

[b]*Alternative affiliation is S. flavovirens.*

3.2. Acidolytic Activity of Isolated Fungi

pH values of broth medium were deceased, and gradual decreasing was observed with increasing of incubation period, and HPLC data indicated that isolated fungi produce a variety of organic acids and oxalic acid was the most present (unpublished data).

3.3. Fungal Biopigment Productin

The fungal isolate (*F. oxysporum*) isolated from red stains on painting and stone surface in produced a pinky pigment was extracellular in nature (**Fig**. 3).

3.4. Effects of Cu and Fe on the Fungal Growth

Current results indicated that $FeSO_4.2.H_2O$ enhanced biomass of *A. japonicus* Saito that was isolated from yellow paintings, tomb of Ankh m b3st. On the other hand, the atomic absorption data indicated that biomass of *A. japonicus* cultured on $FeSO_4\,2.H_2O$ bioaccumulated Fe ions on fungal cell wall at 200 ppm/g of biomass. On the contrary, copper sulphate $CuSO_4.7H_2O$ reduced the growth of all fungal isolates in comparison with control samples and enhanced melanin production. Moreover, the atomic absorption data pointed that *A. japonicus* bioaccumulated Cu ions on the fungal cell walls at 120 ppm/g of biomass.

3.5. Chemical Composition of Thymol and its Color Change at Exposure to UV Light

From the spectral analysis, Mass spectra, ^{1}H NMR spectra and FT/IR, we could conclude that thymol is composed of *β*-cymene (molecular weight is 135) and carvacrol (Fig.4). Moreover, ^{1}H NMR spectra showed signals at δ 2.2 (S, 3 H, CH_3), 3.5 (sept. ^{1}H, CH), 6.26-8.0 (m, 3H, Aromatic), and 9.0 (S, broad, ^{1}H, OH) (Figure.5). Finally, FT/IR pattern showed the presence of phenol groups that exhibited intense bands at 3423 cm-^{1} characterizing phenol groups (Figure.6a).

So from the spectral analysis we could conclude that the chemical composition of thymol is 2-isopropyl-5-methylphenol and its molecular formula is $C_{10}H_{14}O$ (Fig. 6d) and thymol is not in pure form.

After the exposure of thymol to sun rays and UV, it was possible to observe the yellowing of thymol. IR spectra of thymol samples exposed to and UV sun light indicated presence of CH aromatic at 3030 cm^{-1},carbonyl group (C=O), the main bands were at 1723 cm^{-1}, and the fingerprint band of quinon group (ON-O-R) showed at 695 cm^{-1} (Figure.6 b-c).

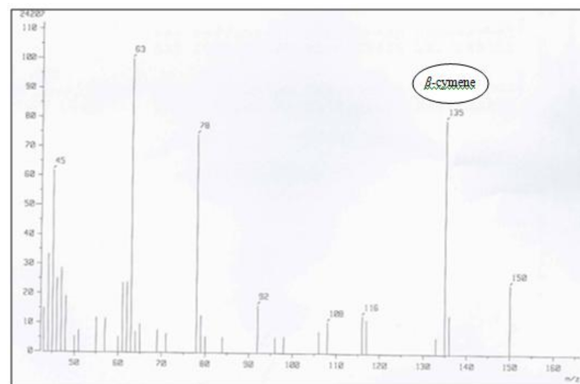

Figure4. *Mass spectra of thymol*

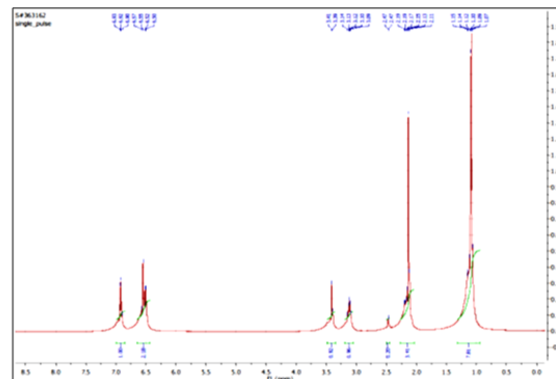

Figure5. *^{1}HNM spectra of thymol*

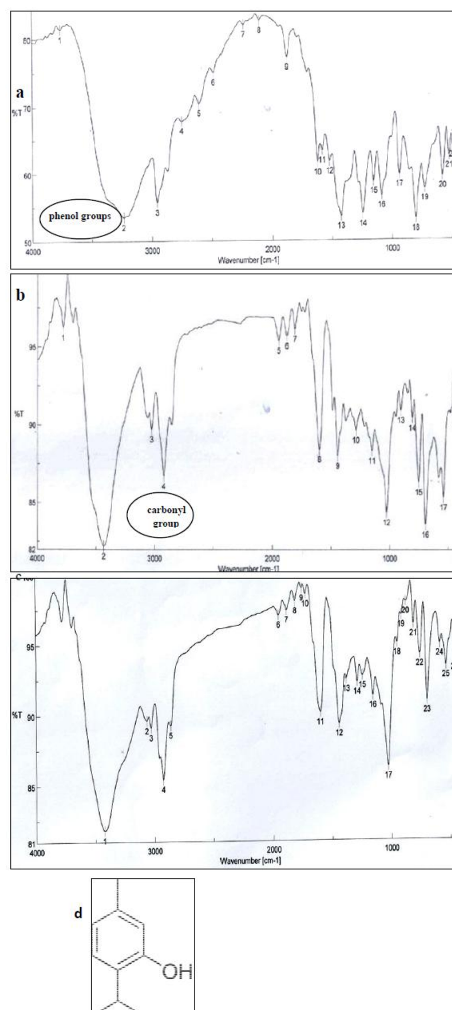

Figure6. *FT/IR spectra of thymol (a) control (b-c) exposed to UV light (d) molecular structure of thymol*

3.6. Effect of Thymol on the Growth of Fungi

Results showed that the effect of thymol on the isolated fungi varied according to its concentration and evidenced that the concentration 10 µg/ml was not effective, but the concentration 100 µg/ml inhibited the growth of the isolated fungi for five days (Table 2), but after ten days, fungi recolonized again over the filter paper discs (Figure.7).

The isolated fungi varied in their resistance profile to tested concentrations of thymol, whereas *Penicillium* sp. was the most resistant, but *A. japonicus* was the most sensitive (Figure.8a).

The other determinant of efficacy of thymol on the isolated fungi was relative humidity (RH), whereas our results pointed out that thymol was more effective in low RH (30%) and less effective in higher RH (70%) (Figure 8b).

Moreover, TEM micrographs pointed out that the treatment of *A. japonicas*, the most present fungus, with different concentrations of thymol delayed spore formation and enhanced deformation of fungal spores (Fig.9).

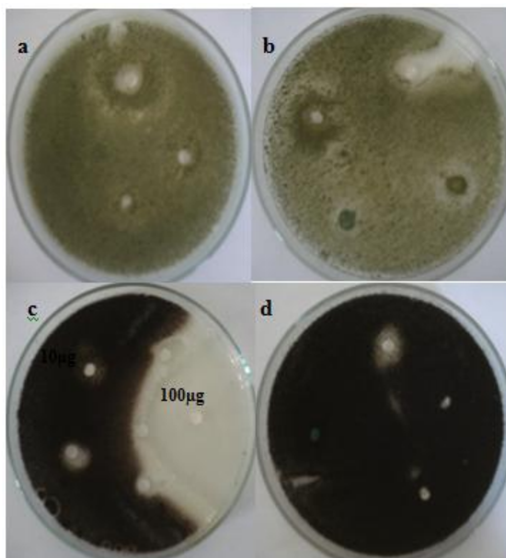

Figure7. *Effect of different concentrations of thymol on (a): penicillum sp. after five days of treatment. (b): after ten days of treatment. (c): A. japonicus after five days of treatment. (d): after ten days of treatment.*

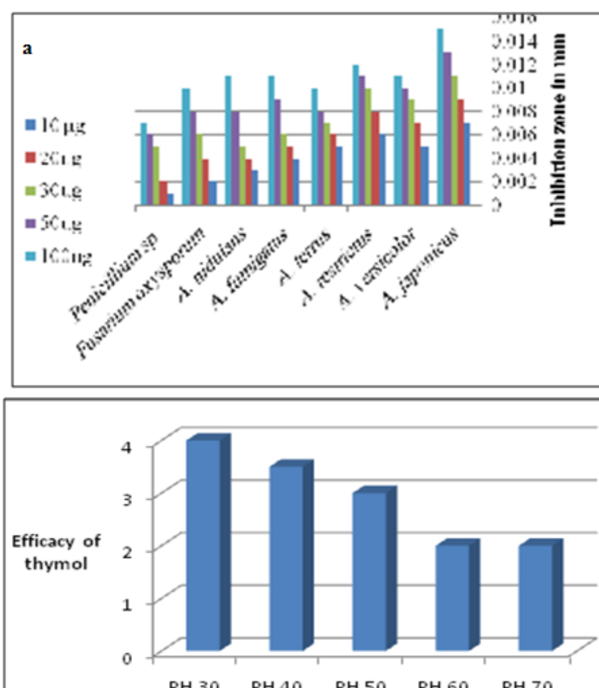

Figure8(a). *Effect of different concentrations of thymol on the fungal isolates. (b) the relationship between efficacy of thymol and relative humidity*

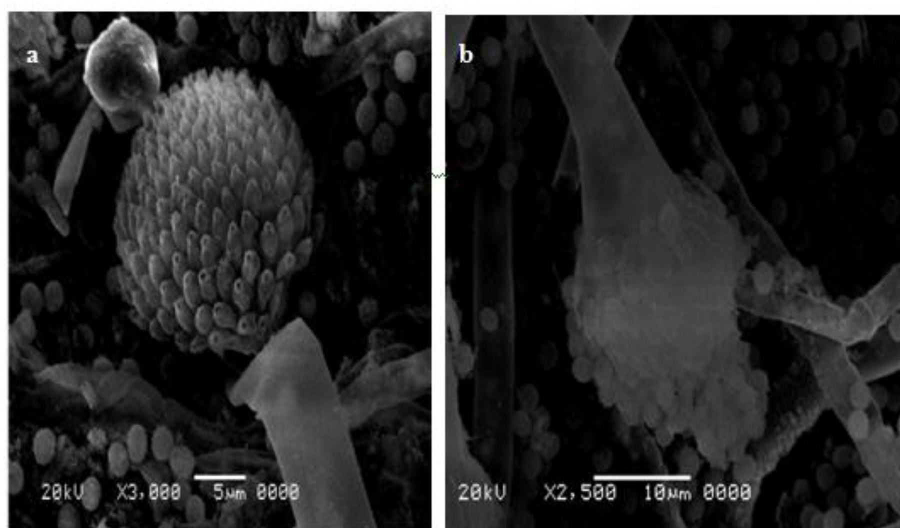

Figure9. *SEM micrograph illustrates spore deformation of A. japonicus by thymol 100 μg (a) control, (b) treated isolate*

3.7. Schematic of Fumigation Chamber

To reduce conservation interventions with direct contact on archeological surfaces, a modified thymol fumigation chamber was used, a modified form of glass test chamber. This chamber was of plexi glass provided with fan to make air circulation within the fumigation chamber and data logger to observe relative humidity and temperature respectively, and humidifier of magnesium sulfate was used to adjust relative humidity to the required level (Figure.10). Our results indicated that using thymol in fumigation within the fumigation chamber prevented re-growth of fungi for a long period of time, more than five months at 20 C and RH 45 %. The amount of thymol required for fumigation ranged from 40-50 g/m^3 and cfu of fungal cells reduced significantly (Table 3).

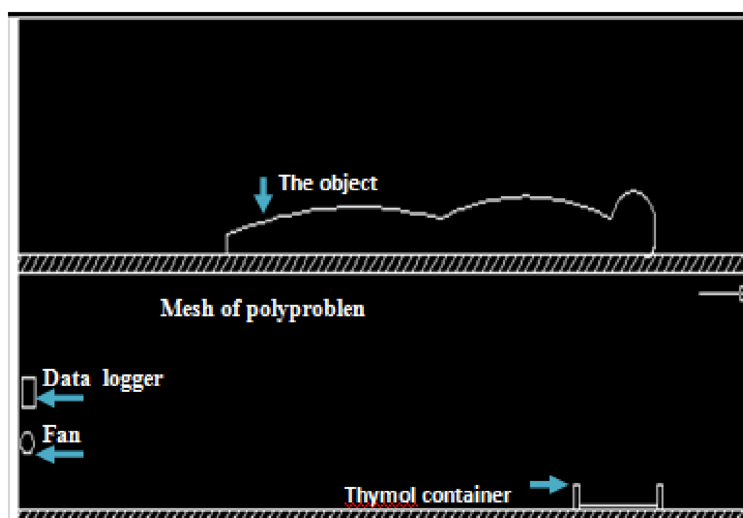

Figure10. *Systematic of fumigation chamber*

Table3. *Effect of different concentrations of thymol on fungal species*

Fungal isolates Inhibition zone (mm) of different Concentration of thymol μg/ml					
Fungal isolates	10	20	30	50	100
A. fumigatus	-	5	7	10	14
A. japonicas	-	10	12	19	25
A.nidulans	-	3	6	8	12
A. restrictus	-	7	9	14	18
A. terrus	-	6	8	12	17
A. versicolor	-	7	10	14	20
F.oxysporum	-	2	4	6	11
Penicillium sp.	-	1	3	5	10

Table4. *Reduction of fungal colonies after fumigation*

Fungal isolate	cfu of viable spores	
	Before	After 10 days
A. fumigates	8×10^{3}	3×10^{1}
A. japonicus	6×10^{2}	1×10^{1}
A.nidulans	3×10^{2}	1×10^{1}
A. restrictus	7×10^{3}	2×10^{1}
A. terrus	5×10^{2}	1×10^{1}
A. versicolor	1×10^{3}	1×10^{1}
F.oxysporum	4×10^{2}	1×10^{1}
Penicillum sp.	2×10^{3}	7×10^{1}

3.8. Effect of Thymol Concentrations on Different Pigments

Finally, FT/IR spectra pointed out that thymol used in fumigation at the concentrations tested in this study had no effect on the investigated pigments such as hematite, limonite, malachite, azurite and black carbon (unpublished data)

4. DISCUSSION

Our results are consistent with the previous studies showing that *Aspergillus* group reported their presence with six species; *A. versicolor, A. japonicus, A. restrictus, A. terreus*, *A. nidulans, A. fumigatus*, *Fusarium oxysporum* and *Penicillium* sp. were the most present species in the deteriorated mural paintings (Sterflinger, 2010; Garg et al. 1995; Guglielminetti et al. 1994) and were biodeteriorative agents for various monuments (Biswas et al. 2013). On the other hand, it was well documented that *Aspergillus* was the most present deteriorating agent for mural paintings within the temple of Abydos, Upper Egypt, and strongly associated with seasonal environmental fluctuations (El Sharonuny et al. 2000a).

Our finding pointed out that there is a difference between fungi isolated from air, limestone surfaces and paintings that indicated the selectivity of fungi involved in deterioration of paintings and stone surfaces within the investigated tombs (Karbowska-Berent et al. 2011).

Current results showed that *F. oxysporum*, produced a pinky pigment on Czapek-Dox plates that causes disfiguration and aesthetic damage of colonized mural paintings and stone surfaces with irreversible stains; in particular these biogenic pigments were extracellular in nature and mostly are resistant to chemical and enzymatic degradation (Ravikumar et al. 2012; Szezpanowska and Lovett 1992). Involvement of fungi in disfiguration of paintings and stone surfaces was put onto the evidence, since the black, green and red stains on wall paintings from ancient tombs in Japan were attributed to the fungal colonization (Garg et al. 1995).

In this study, it was observed that *A. japonicus* (46%) was the most isolated fungus because it is a thermophilic fungus and collecting of samples was carried out in the summer months (June-September) characterized with high temperature more than 50 °C (da Silva et al. 2012).

Current results indicated decreasing of pH values with increasing of incubation period, and HPLC data showed that isolated fungi are acid producers and the most produced acids are oxalic, citric and glucconic acids, that cause irreparable chromatic alteration of colonized paintings (Biswas et al. 2013; Ravikumar et al. 2012).

Current results indicated that $FeSO_{4}.2H_{2}O$ enhanced biomass of *A. japonicus* Saito isolated from yellow color paintings with limonite from the tomb of Ankh m b3st. On the other hand, atomic absorption data indicated that the biomass of *A. japonicus* cultured on $FeSO_{4}\,2.H_{2}O$ bioaccumulated Fe ions on fungal cell walls. This may be explained by the matter of fact the most microorganisms need iron for enzyme function because it is considered the key component of cytochromes and electron carrying proteins, which play a major role in the cellular respiration in subsurface environments (Abdel-Haliem et al., 2013b; Kalinowski et al. 2000).

Furthermore, data obtained from the atomic absorption analyses pointed out that *A. japonicus* bioaccumulated Cu ions on the fungal cell walls at 120 ppm/g of biomass. These results were confirmed those of Milanisi et al. (2006). Also, it was demonstrated that *A. juponicus* produce citric acid and oxalic acid. (Domsch et al., 1980).

Moreover,, our finding pointed out that copper sulphate $CuSO_4.7H_2O$ reduced the growth of all isolated fungi and enhanced melanin pigment, that may be attributed to the matter of fact the production of melanin is a defense mechanism against adverse environmental conditions such as heavy metals, hyper salinity, and irradiation whereas melanin acts as a shield preventing access of these heavy metals into microbial cell (Abdel-Haliem et al., 2013b).

Also, it was observed that fungal isolates from blue color was little (1 isolate), that may attributed to the matter of fact that fungi are less resistant to copper based pigments such as azurite blue (Caeser-Tonthat et al. 1995), the blue pigment detected in the investigated mural paintings at Tell Basta (Abdel-Haliem et al., 2013b).

With regard to the chemical composition of thymol, Mass spectra data indicated that thymol is not a pure form, but it is a mixture of thymol, *β*-cymene and carvacrol. In supporting these results, Garca-Risco et al. (2011) and Sirvaityte et al. (2012) reported that thyme oil, the major source of thymol, is composed mainly of thymol (38%), *β*-cymene (8.64%) and carvacrol (10.11%) in addition to terpenoids such as menthol, camphor and phytoln and the ratio of these components varied according to plant source where this oil was extracted.

On the other hand, ^1H NMR spectra revealed that thymol has simple organic structure is 2-isopropyl-5-methylphenol, with molecular formula $C_{10}H_{14}O$ (Garcia-Risco et al. 2011; Walentowska and Foksowicz-Flaczyk 2012).

Moreover, FT/IR spectra showed the presence of phenol groups that have a strong antimicrobial activity against a wide range of microorganisms of fungi and bacteria either gram positive or gram negative (Sirvaityte et al. 2012; Soumya et al. 2011; Hu et al. 2008).

Current results indicated that the exposure of thymol to sun rays and UV lead to the yellowing of thymol, and IR spectra of exposed thymol samples indicated presence of CH aromatic, carbonyl and quinon groups. So, we could conclude that yellowing was attributed to the photochemical oxidation of phenol groups where thymoquinone or dithymoquinone have increased carbonyl absorption in the infrared spectrum (Sequeira et al. 2012; Daniels and Boyd 1986).

Moreover, the effect of thymol on the isolated fungi varied according to its concentration and fungal species since our results indicated that the concentration 10 µg/ml was not effective, but the concentration 100 µg/ml inhibited the growth of isolated fungi for ten days. Our finding agreed with those obtained by Haines and Kohler (1986) who tested effect of thymol against *Aspergillus niger*, *Aureobasidiumpu pullulans*, *Cladosporium* sp., *Chaetomium* sp., *Fusarium* sp., and *Penicillium* sp. colonizing archives and leather clothes and found that *A. niger* was the most resistant, but *Fusarium* sp. was the most sensitive to different thymol concentrations.

On the other hand, it has been established that thymol was effective against fungi colonizing a wide range of cultural heritage objects such as leather bindings and paper documents and *A. niger*, *Emericella nidulans*, *A. solani*, *Penicillium rotatum*, *Fusarium oxysporum*, *Chaetomium globosum* were the most present (Velikova et al., 2011).

From culture dependent and independent techniques, it was observed that microbiota are composed of different fungal species, and the isolated fungi varied in their resistance profile to tested thymol concentrations, since *Penicillium* sp. was the most resistant, but *A. japonicus* was the most sensitive at 100 µg. This an opinion also held by Wild (1954).

SEM micrographs indicated that treatment of *A. japonicus*, the most present fungus in the investigated tombs, with thymol 100 µg/ ml delayed the fungal spore formation. This result explained the reason of mortality of treated fungi with thymol and was in agreement with the results obtained by Veilkova et al. (2011) showing that the treatment of *A. niger* with thymol caused deformation of fungal mycelium and significant changes of fungal spore were observed, also Bennis et al. (2004) reported TEM micrographs of *Sacchromyces cervisiae* treated with thymol showing significant deformation and apparent cracks in the treated yeast cells. Also, Walentowska and Foksowicz-Flaczyk (2012) found that thyme oil, the main source of thymol, completely inhibited the mycelial growth of *Aspergillus flavus* and exhibited a broad fungitoxic spectrum against *Aspergillus niger*, *A. fumigatus* and *Alternaria alternata*.

Results derived from this study pointed out that thymol acted as fungistatic, where regrowth of treated fungi was observed after 10 days. This in agreement with Craig (1986), who inoculated filter paper samples with four cellulolytic fungi species, and treated with thymol by fumigation (20 g / m^3), with a constant temperature at 37°C for 3 days, and fungal growth appeared after 10 days of the treatment. On the other hand, the inhibitory effect of thymol on fungi was a temporarily effect and it was found that thymol 100 µg/ml delayed the fungal growth of *Penicillium citrium* for up to 9 days, but this fungus recolonized again (Vazuez et al. 2001). Furthermore, Gustafson et al. (1990) stated that thymol was not totally fungicidal for the fungal species tested, since the fungal spores were not totally eliminated, so it could be used in fumigation.

Our finding indicated that thymol in fumigation prevented recolonization of fungi for a long period of time, more than five months. These results are similar to those of Haines and Kohler (1986) where thymol was used for fumigation within fumigation chambers and could reduce the number of viable spores of fungi after the fumigation process was finished, because antifungal vapors had prolonging thymol effect on the treated fungi (Sequeira et al., 2012; Daniels and Boyd 1986).

Current results indicated that thymol vapors were effective decontaminating colonized objects of cultural heritage and this may be attributed to the continuous presence of thymol vapors within the exhibition cases that prevent growth of viable fungal spores (Rakotoniainy and Lavedrine 2005; Craig 1986), that allows a better penetration of the active compounds into the cultural heritage objects than liquids (Nugari 2003), and this efficacy of fumigation should be enhanced by low vapor pressure of thymol (Isabell, 1997).

Our finding indicated that amount of thymol required for fumigation ranged from 40-50 g/m^3, and the fumigation process lasted from 3 days to 12 days, this in agreement with Craig (1986) reported that weight of thymol required for fumigation ranged from 1 to 90 g/m^3, and the exposure time ranged from 24 h to 3 weeks.

On the other hand, it has been established that thymol fumigation could be used as a preventive conservation agent for sensitive organic materials stored in humid cases (Isbell, 1997).

Furthermore, FT/IR spectra indicated that thymol concentrations fumigated in this study had no effect on the investigated pigments such as hematite, limonite, malachite, azurite and black carbon. This agree with results obtained by Isabell (1997) who reported that thymol solution did not cause any changes to the treated pigments, but caused discoloration of inks in the treated manuscripts due to oxidation of phenol, and this phenomenon was well documented for organic pigments such as rose madder that was affected by exposure to thymol (Daniels and Boyd 1986).

5. Conclusion

Fungi involved significantly in biodeterioration of mural paintings within the tombs at Tell Basta by forming irreversible microbial stains and chromatic alterations by acids. Thymol was used in decontamination of fungi colonizing mural paintings and showed to be fungistatic since fungi recolonized after ten days of the treatment, so the fumigation method was used. The fungal resistance profile varied according to the fungal species, since *A. japonicus* was the most sensitive to thymol at 100 µg/ml, (inhibition zone 25 mm), but *Penicillum* sp. was the most resistant at the same concentration (inhibition zone 10 mm). Using thymol at 40-50 g/m3 for 3-12 days in the fumigation process reduced the numbers of fungi colonizing the tested objects of cultural heritage significantly. Thymol vapors were more effective in lower humidity than higher one. Finally, FT/IR spectra confirmed that thymol caused changes neither for the tested pigments nor for the surface appearance.

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