EVALUATING THE ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS IN THE CONSERVATION OF MURAL PAINTINGS

Mayssa D. Albasil
Conservation Department, Faculty of Archaeology
Luxor University, Egypt

Gamal Mahgoub, Abeer ElHagrassy
Conservation Department, Faculty of Archaeology
Fayoum University, Egypt

Amany M. Reyad*
Botany Department, Faculty of Science
Fayoum University, Egypt

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1. Introduction

Mural paintings are considered as painted history on walls, which provide us with information about our ancestors; therefore, mural paintings bear essential components of cultural heritage [1-2]. Unfortunately, due to deterioration factors, these murals can become discolored and degraded especially as a result of the growth and activity of microbes [3]. Fungi are recognized as the most effective microorganisms at colonizing mural paintings [4].

Mural paintings consist of organic and inorganic materials and are components that can be used as a nutrient source for microbial colonization [5]. Microbial biodeterioration, especially fungal deterioration, occurs on mural paintings when conditions such as temperature and relative humidity are favorable to the growth of fungi and spores [2].

Microbes generally cause aesthetical, physical, and chemical deterioration on different mural paintings [6]. Aesthetical deterioration often appears as discoloration on paintings, destroying their aesthetics, stability, and the original intent of beautifying. This discoloration can be caused by the colored pigments (black, green, pink, grey, white, and purple) produced by some fungi as a result of their metabolic activity.

Fungi can cause the physical deterioration of mural paintings triggered by the growth of hyphal networks through the pore space system; the process involves the filamentous structures of the fungi penetrating through the mural painting layers, the penetration also possibly being favored by turgor pressure inside the hyphae, leading to cracking, fissures, paint blisters and detachment of the paint layer [7]. Chemical deterioration occurs due to microbial metabolic products (including organic and inorgan-

* Corresponding author: amr01@fayoum.edu.eg

ic acids and mycotoxins) and extracellular enzymes, which react chemically with mural painting components leading to mineral dissolution and structural changes in the components of the mural painting [2].

Moreover, microbes can release spores, hypha fragments, metabolites, toxins, and allergens into the aerosol of the indoor environment of the cultural heritage, affecting human health at workplaces and causing serious respiratory infections, such as bronchial irritation and allergies [8]. Several traditional methods are used for controlling and treating the fungal deterioration of mural paintings, including mechanical methods using brushes, scrapers, and scalpels for the mechanical removal of the mycelia, physical methods using UV, IR, γ-Rays, X-rays, etc. which are considered short-term treatments and may lead to staining, whitening, or brittleness of historical paintings [9]; chemical methods are also employed, using different biocides to inhibit microbial growth on the painted surfaces. However, these biocides have a high toxicological impact on the environment and human health and some of them have been banned over time [9]. Unfortunately, these controlling methodologies (traditional, physical and chemical) have numerous disadvantages, for instance, toxicity; environmental hazards; higher costs; changing some of the properties of heritage objects, etc.

In this context, natural products could be a favorable alternative to traditional methods, in particular, essential oils (EOs) from plants which are recognized as an eco-friendly, efficient, low cost, and easy to use approach for the control of various types of microorganisms [1]. EOs (also called volatile oils) are aromatic oily liquids obtained from various plants and extracted from particular parts of the plant, such as leaves, stems, flowers, seeds, twigs, bark, wood, fruits, and roots [10]. The antimicrobial activity of EOs is due to their various components including terpenes, aldehydes, alcohols, esters, phenolics, ethers, and ketones [10].

Various studies have been done on the antimicrobial activity of different essential oils in the field of cultural heritage conservation, but little is known about their application in the conservation of mural paintings [11]. It is quite difficult to compare the results of various studies, as the composition of the essential oils varies greatly depending on the kind and age of the plants, the geographical region, and methods of extraction of the oil [10].

This research aims to evaluate the antimicrobial activity of two essential oils (lemon oil and thyme oil) on the growth of fungi and bacteria isolated from mural paintings in the tomb of Khety (Tomb No. 17) at Beni Hassan, Egypt, and assay the effect of these essential oils on the pigments of the mural paintings.

2. Material and Methods

2.1. Mural painting case study

In this research, black biofilms and fragments of the mural paintings in the tomb of Khety (Tomb No. 17, 11th Dyn.) were collected (Figure 1). It is one of the Middle Kingdom rock-cut tombs at Beni Hassan cemetery in Egypt, cut in the cliffs overlooking the east bank of the River Nile; its mural paintings were executed using a tempera technique. Each of the decorated rock-cut tombs at the site display highly detailed scenes of daily life (e.g., agricultural tasks, hunting and fishing, workshop activities, funerary rites, etc.).

Figure 1. (A) Mural painting of the Khety tomb (Tomb No. 17, 11th Dyn.) at Beni Hassan, Egypt; (B) left side of the west wall with visible black biofilms on the chromatic layer.

2.2. Microbial Isolation and identification

Fungi on the mural painting were isolated by direct swabbing from the spots showing signs of microbial deterioration; swabs were taken over the four seasons in January, April, August, and November [3]; sterile cotton swabs were wiped over paintings then transferred to the laboratory in sterile tubes on the same day of collection. For fungal isolation, swabs were cultured directly on previously sterilized yeast extract agar dishes. The isolated fungi were morphologically identified.

On the nutrient agar dishes, swabs were cultured for bacterial isolation. For the molecular identification of the most predominant bacterial isolate, the genomic DNA was extracted, and the PCR blend was made as described by Hemida and Reyad [12]. The sequencing of the amplified fragments was completed at GATC Biotech, Constance, Germany. DNA Sequences were aligned using the NCBI Data Base (www.ncbi.nlm.nlh.gov). The phylogenetic tree was established using a neighbor-joining technique using TREEVIEW software (1.6.6) derived from gene sequences of 16SrRNA of some phylogenetic strains close to the isolated strain.

All these dishes were sealed with Parafilm and incubated at 28±2°C for five days. The colonies of fungi that appeared in the dishes after incubation were then purified in new Petri dishes. A pure culture of each colony type was obtained on each Petri dish. Yeast extract media (01497, Sigma Aldrich) was used for fungal isolation and purification. Based on the samples collected, the predominant three fungal isolates and one bacterium were chosen to test two essential oils to evaluate their efficacy.

2.3. Essential oils

Lemon oil and thyme oil were tested for their antimicrobial activity. The oil extracts were prepared at Cornell University, New York. *Citrus limon* (Lisbon variety) leaves were

used to extract the lemon essential oil. The fresh Citrus leaves were shade-dried at room temperature 35 /40°c for 72h and powdered in a blender. 100g of the powder was subjected to hydro-distillation for 6h using Clevenger apparatus. The thyme (*Thymus vulgaris*) leaves were collected and dried for five days at room temperature then freeze-dried; 100g of the powder was obtained by steam distillation from leaves under natural conditions. The two extracted essential oils were kept in dark bottles (Brown bottles) at 4°C until further investigation. Dimethylformamide (DMF), toluene, and ethanol 70% were chosen to dilute the studied EOs to determine the most effective solvent.

2.4. Mural painting structure

A small fragment was taken from the mural painting, the support, each pigment and the ground layers for analysis and examination.

X-Ray Diffraction Analysis (XRD)

An XRD Bruker company model D8 was used for compound identification of the samples; it includes reflectometry, featuring high-resolution diffraction, in-plane grazing incidence diffraction (IP-GID), in addition to small-angle X-ray scattering (SAXS).

Scanning Electron Microscope (SEM) and EDS

The examination and analysis of the mural painting samples were carried out by a Bruker SEM (Berlin - Germany) equipped with energy-dispersive spectrometry (QUANTAX EDS) featuring the XFlash® 6 detector series with active areas from 10 to 100 mm^2 combined with the modular ESPRIT software.

Fourier transmission infrared spectroscopy (FTIR)

FTIR analyses were executed using a Nicolet Nexus spectrophotometer (Washington, USA), coupled with a Nicolet Continuum Fourier transmission infrared spectroscopy microscopy equipped with an HgCdTe detector cooled with liquid N2. Spectra were recorded by a Graseby-Specac diamond cell accessory in transmission mode between 4000 and 700 cm-1.

Raman spectroscopy

Raman spectra were recorded using a LabRAM HR Evolution Raman spectrometer (HORIBA JOBIN YVON) equipped with multi laser capability, a true confocal microscope, a high-performance Raman spectrometer, multiple detectors and powerful software (LabSpec 6 software).

2.5. Preparing replica and artificial aging

In order to evaluate the effectiveness of the essential oils chosen for the study, replicas of the mural paintings (executed using a tempera technique) were prepared and aged. Ten cycles of thermal aging in an oven at 60ºC were carried out for eight hours followed by eight hours at room temperature [13].

2.6. In vitro antimicrobial assay

The antifungal activity of EOs was tested using the disc-diffusion method under sterile conditions. In this well-known method, agar plates were prepared using Petri

dishes (90 mm diameter), each Petri dish containing 20 ml of autoclaved microbial agar media (potato dextrose agar for fungal isolation and nutrient agar for bacterial isolation). All plates were inoculated with bacterium (*Alcaligenes faecalis*) and a spore suspension of the three tested fungi (*Aspergills Niger, Penicillium Cyclopium, Fusarium Solani),* which was spread over the entire surface of the agar plates by swabbing. Filter paper discs about 6 mm in diameter (Double Rings® filter paper) were impregnated with the EOs at different concentrations (5%, 10%, and 15%), and 70% ethyl alcohol was used for diluting the essential oils, then placed on the agar surface [14].

Negative control plates were performed by wetting the filter paper discs with 70% ethyl alcohol. Plates with no oils were used as control plates. The test was performed in duplicate. All dishes were sealed with Parafilm and incubated at 28±2°C until the microbial growth fully covered the surface of the medium in the control plates. After incubation, microbial growth was observed and the halo diameter (mm) of inhibition growth zones around the disc was measured.

2.6.1. In vitro antimicrobial activity of EOs on replicas

The most effective concentrations of the essential oils obtained from the above experiment on agar plates were tested on replicas for their antimicrobial activity and probable effect. Some replicas were treated with EOs at the most effective concentrations of 10% and 15% and 70% ethyl alcohol used for dilution; the other set of replicas was left untreated as a control. Replicas were inoculated with freshly prepared bacterial suspension (1 x 10^7 CFU/ml) and spore suspensions (1 x 10^6 spores/ml) of the three tested fungi by spreading the suspensions uniformly on the surface under sterile conditions. All inoculated replicas were put in sealed containers and incubated for two months and were examined every week.

2.7. Colorimetric measurements

Colorimetric measurements were performed using a portable spectrophotometer, the Spectro densitometer by eXact X-Rite Switzerland, to measure the different colors of the replicas before and after being treated with the oils to identify any probable color changes that might have resulted after treatment.

2.8. Gas chromatography/mass spectrometry (GC/MS) analysis of the EOs

The GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra / ISQ Single Quadrupole MS, TG-5MS fused silica capillary column. For GC/MS detection, helium was utilized as the carrier gas at a constant flow rate of 1mL/min, and an electron ionization apparatus with ionization energy of 70 eV was used. The temperature of the injector and MS transfer line was fixed to 280°C. The oven temperature was programmed at an initial temperature of 40°C (held for 3 min) to 280°C as a final temperature at an increasing rate of 5°C/min. A percentage of the relative peak area was used to explore the quantification of all the detected components. The chemicals were tentatively identified by comparing their respective retention times and mass spectra with those of the National Institute of Standards and Technology (NIST), WILEY library database in the GC/MS instrument.

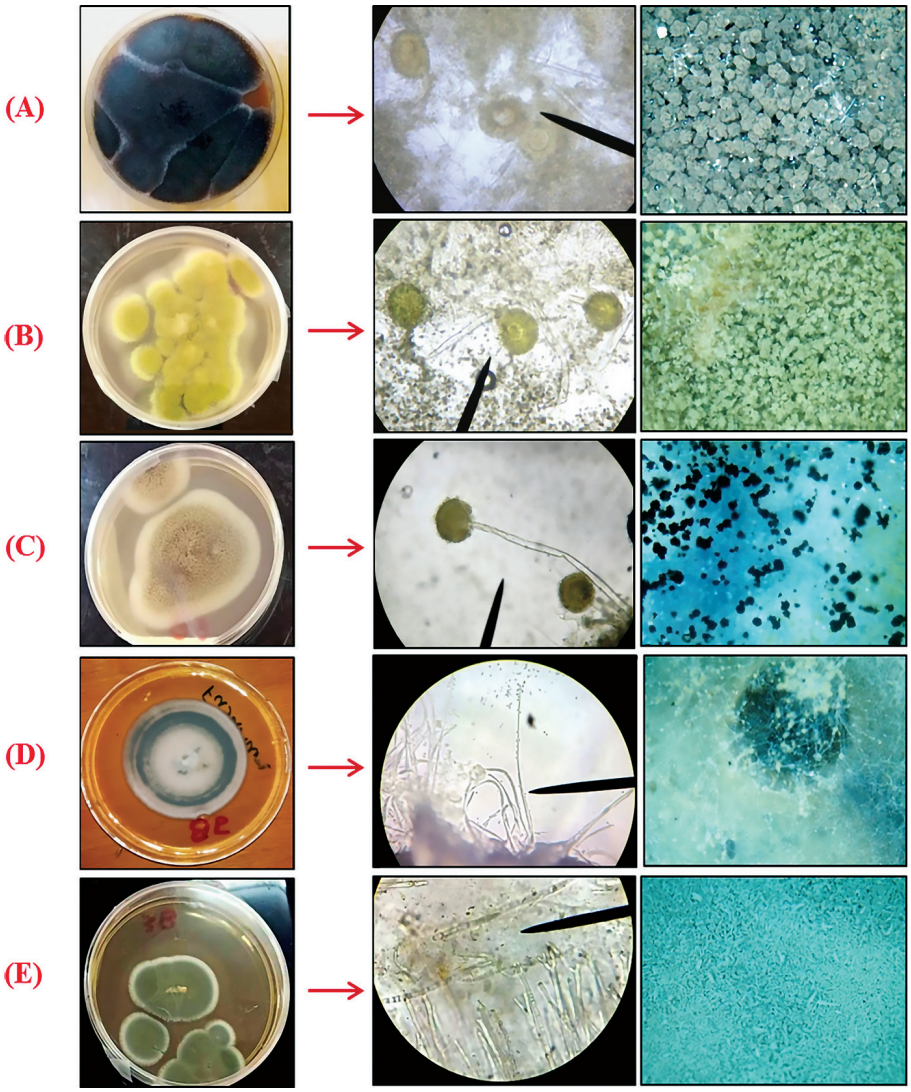

Figure 2. Morphological appearance of the fungi identified in the tomb: (A) Aspergillus niger; (B) Aspergillus flavus; (C) Rhizopus stolonifer; (D) Fusarium solani; E) Penicillium cyclopium.

2.9. Antimicrobial assay of thymol and citral

Citral (96%) and thymol (98.5%) were purchased from Sigma-Aldrich, and ethanol (70%) was used for the preparation of 5, 10, and 15% of the two compounds. The common disc-diffusion method was used for antimicrobial detection [14].

3. Results

3.1. Microbial identification

Based on the swabs taken over the four seasons in (January, April, August and November), the data shows that deterioration of the tomb was caused by the effects of five fungi: *Aspergillus niger, Aspergillus flavus, Fusarium solani, Rhizopus stolonifer, Penicillium cyclopium*) (Figure 2). It can be seen clearly from molecular identification that the tested bacterium was included in the genus *Alcaligenes* and closely related to the species *faecalis*. It showed the highest sequence similarities with the *Alcaligenes faecalis* strain NBRC 13111 (Figure 3).

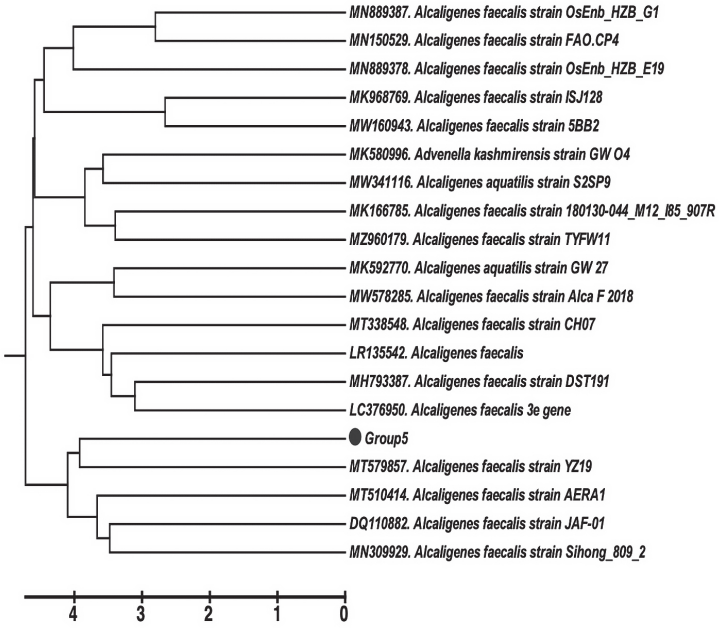

Figure 3. Phylogenetic dendrogram obtained by the distance matrix analysis of 16S rRNA sequences, showing the position of the isolated bacterium strain among phylogenetic neighbors. The dark circle indicates the position of our isolated strain.

3.2. Dilution of the essential oils

The tested essential oils were diluted with dimethylformamide (DMF); they mixed very well and formed a homogeneous mixture. However, it was noticed that dimethylformamide affected the color of the tested EOs, as the color of the liquids became darker in color and over time the color change increased (Figure 4).

When the tested EOs were diluted with toluene, they did not mix and form a heterogeneous mixture (Figure 5). By diluting in ethanol 70%, it was noticed that ethanol 70% did not affect the color of the tested oils, and it mixed very well with the oils in the first hour, but after a few hours, a thin layer of the oils appeared on the surface (Figures 6 and 7).

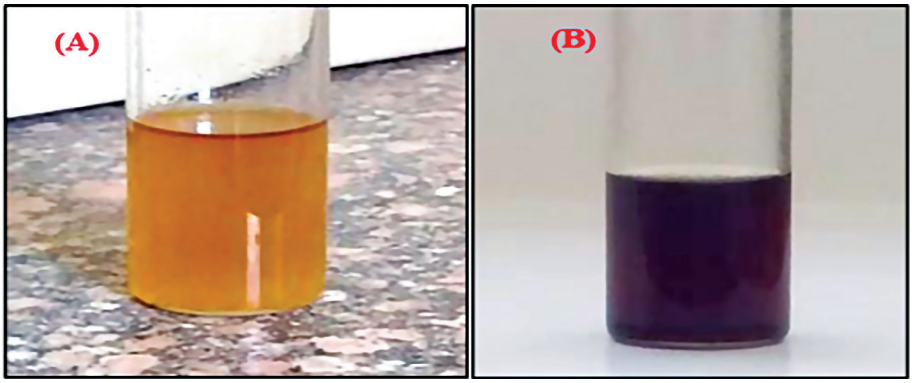

Figure 4. Color change of thyme EO when diluted with DMF. A) Directly after dilution; B) after 7 days.

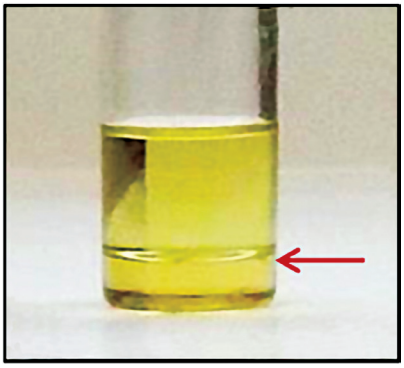

Figure 5. Thyme EO diluted with Toluene.

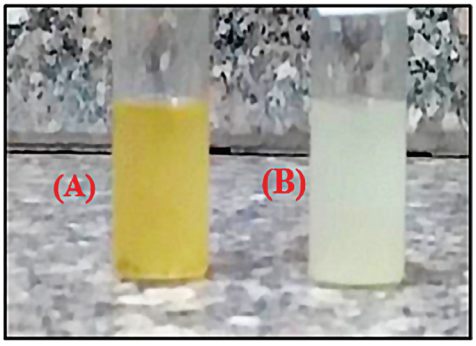

Figure 6. EOs directly after diluting with ethanol 70%. A) Thyme; B) lemon.

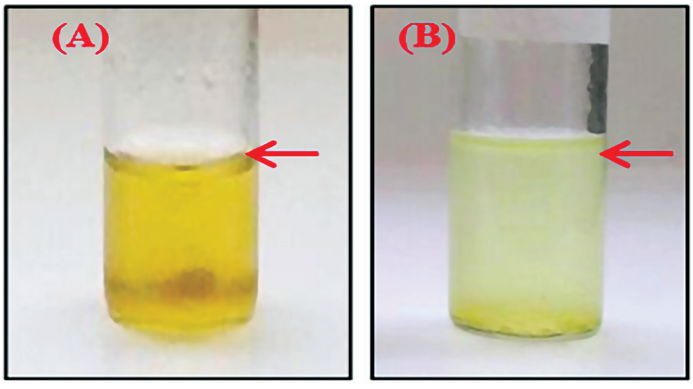

Figure 7. EOs diluted with ethanol 70% after a few hours. A) Thyme; B) lemon

3.3. Analysis of Mural painting structure (support, pigments and media)

3.3.1. X-Ray Diffraction Analysis (XRD)

As shown in Figure 8, the XRD analysis of the support showed 83.9% calcite ($CaCO_3$) and 16.1% magnesium calcite ($CaMg(CO_3)_2$).

For the ground layer, the XRD analysis indicated 69% calcite ($CaCO_3$), 30% anhydrite ($CaSO_4$), and 1% quartz (SiO_2).

XRD analysis of the painted layer showed that the red pigment sample consists of (96%) calcite ($CaCO_3$), 2.8% quartz (SiO_2), and 1.2% hematite (Fe_2O_3); the yellow pigment sample consists of 61.1% goethite (FeO(OH)), 2.30% calcite ($CaCO_3$), 5.2% anhydrite ($CaSO_4$), and 5.3% quartz (SiO_2).

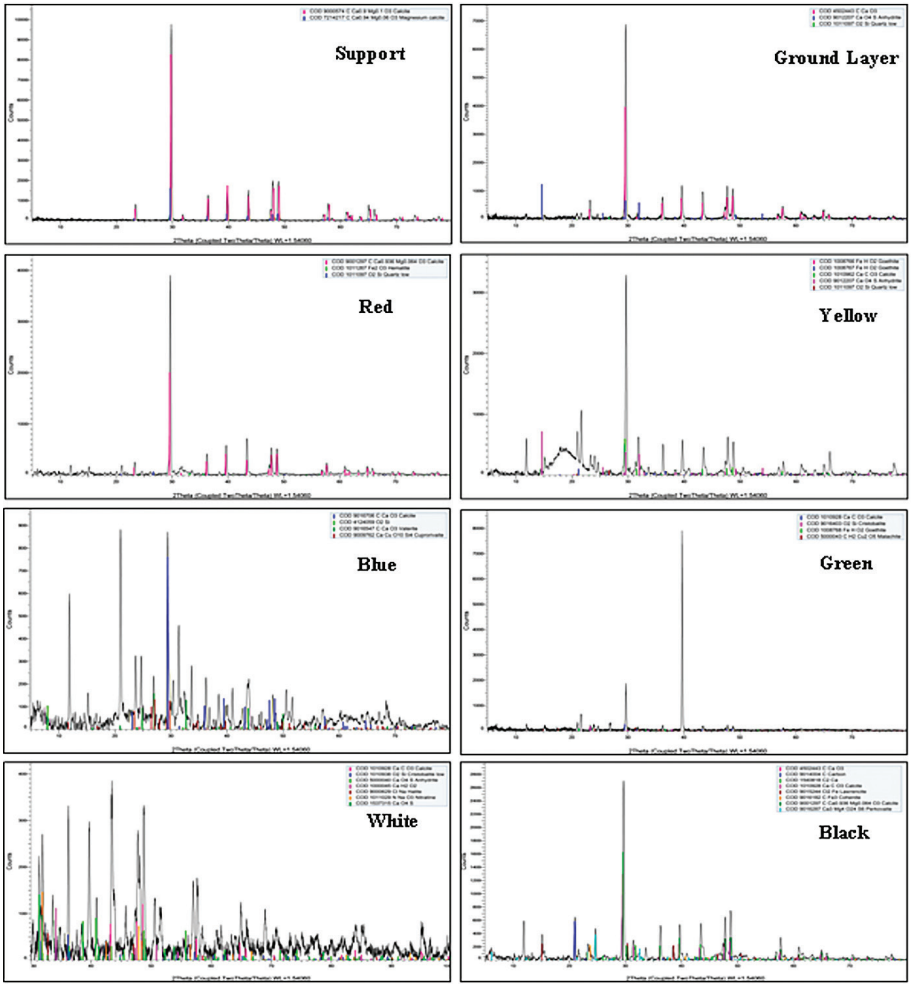

Figure 8. XRD pattern of the mural painting samples.

The ground layer results showed 51.2% calcite ($CaCO_3$), 28.3% vaterite ($CaCO_3$), 18.8% cuprorivaite ($CaCuSi_4O_{10}$) the basic constituent of Egyptian blue, and 1.6% quartz (SiO_2) for the blue pigment sample. XRD analysis of the green pigment sample showed that it consists of 35.9% calcite ($CaCO_3$), 32.7 malachite ($Cu_2CO_3(OH)_2$), 20.1% cristobalite (SiO_2), and 11.3% goethite (FeO(OH)) and showed 23.8% anhydrite (CaSO4), 23.6% calcite ($CaCO_3$), 22.7% nitratine (NaO_3), 13.3% cristobalite (SiO_2), 9.1% calcium sulphate ($CaSO_4$), 5.4% calcium hydroxide ($Ca(OH)_2$), and 2.2% halite (NaCl) for the white pigment. XRD analysis of the black pigment sample showed that it consists of (38.8%) calcite ($CaCO_3$), 27.6% Dolomite ($CaMg(CO_3)_2$), 12.2% carbon (C), 8.9% calcium carbide (CaC_2), 7% perkovaite, 3.5% lawrencite and 1.9% cohenite.

3.3.2. Scanning Electron microscope (SEM) and EDS

The analysis showed that the support consists of Ca, C, O the elements of calcite ($CaCO_3$), and Ca, Mg, C, O the elements of dolomite ($CaMg(CO_3)_2$). The analysis showed that the ground layer consists of Ca, C, O, the elements of calcite ($CaCO_3$); Si, O, the elements of quartz (SiO_2); and Ca, S, O, the elements of anhydrite (CaSO4). For the painted layer as shown in Figure 9, the red pigment sample analysis showed

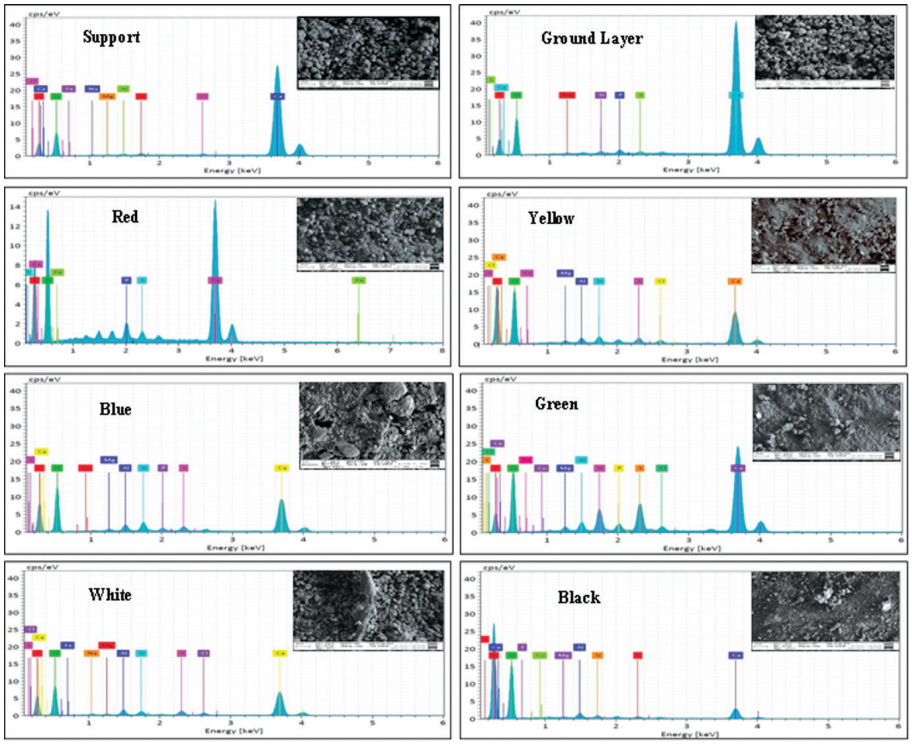

Figure 9. EDS-SEM pattern analysis of the pigments.

the presence of O, Fe, the basic constituents of hematite (Fe_2O_3) and the yellow pigment sample analysis showed the presence of O, Fe, the basic constituents of goethite (FeO(OH)). The blue pigment sample analysis showed the presence of O, C, Ca, Si, Cu, the basic constituents of Egyptian blue ($CaCuSi_4O_{10}$), the green pigment sample analysis showed the presence of O, C, Cu, the basic constituents of malachite ($Cu_2CO_3(OH)_2$), the white pigment sample analysis showed the presence of O, C, Ca, the basic constituents of calcite ($CaCO_3$), and the black pigment sample analysis showed the presence of a large amount of C and traces of other elements (Ca, Si, Mg, Cu, S, Al) indicated the use of charcoal as a source of the black pigment. SEM examination of the archeological pigment samples showed the presence of fungal hyphae between grains (Figure 10).

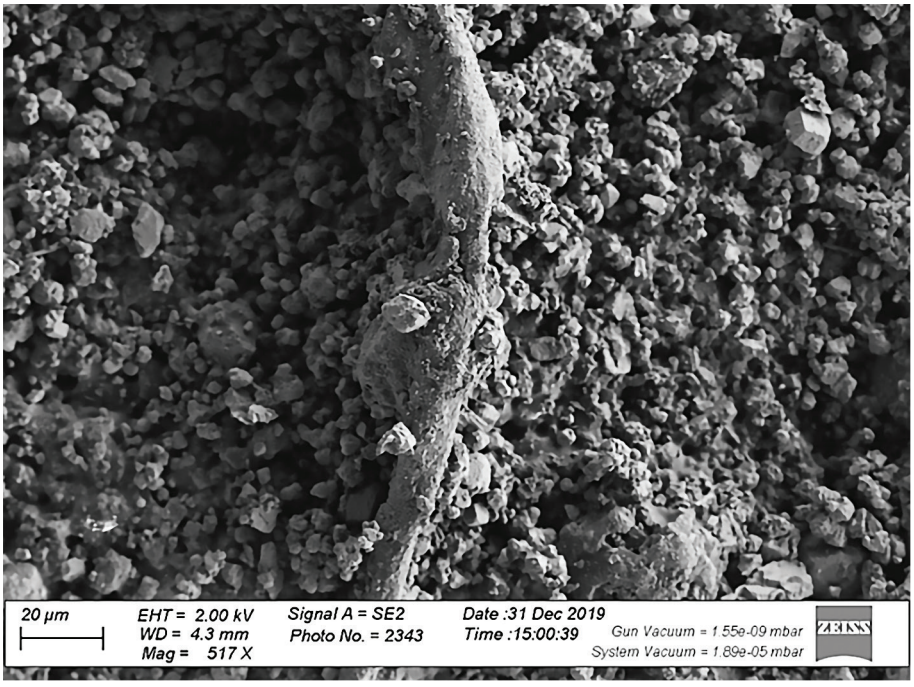

Figure 10. SEM micro-image showing the penetration of fungal mycelium between mural painting grains.

A pigment fragment was analyzed by FTIR to identify the binding medium that was used in the painting process. FTIR spectra showed that Arabic Gum was used as a binding medium. The sample was characterized in the presence of the activated group as: (O-H) in 3423.99, (C-H) in 2975.62, (O-H) in 1624.73, (C-H) in 1424.17 and (C-O) in 874.56 (Figure 11). Another sample was analyzed by Raman spectroscopy to identify the medium used in the painting process. Raman spectra showed that Arabic Gum was used as a binding medium. The sample was characterized in the presence of the activated groups for Arabic Gum.

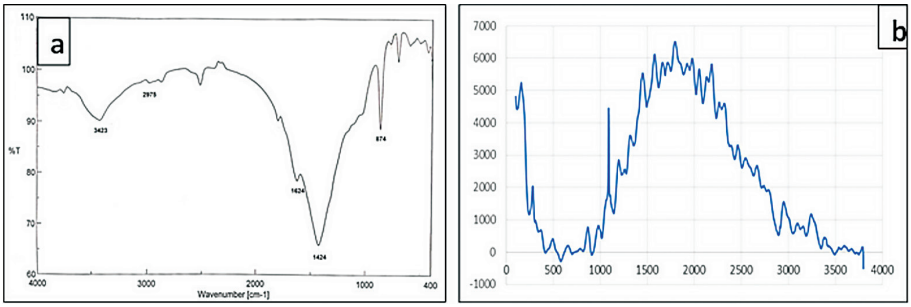

Figure 11. Arabic Gum is used as a binding medium of the pigments. a) FTIR pattern; b) Raman spectra.

3.4. In vitro antimicrobial assay

The results of the antimicrobial activity of the two studied EOs against *Aspergillus niger*, *Penicillium cyclopium, Fusarium Solani* and *Alcaligenes faecalis* on agar plates, revealed that the two oils had an inhibitory effect on the growth of studied microbes, and that an increase in oil concentration leads to an increase of inhibition zones (Table 1). The inhibitory action results in the appearance of a transparent halo (inhibition growth zone) around the filter paper disc impregnated with the studied essential oils.

Results revealed that thyme oil has a more powerful antifungal effect than antibacterial activity. It was observed that 5% thyme oil has neither an antifungal effect against *Fusarium Solani* nor against *Alcaligenes faecalis* bacterium, while the average diameter

Table 1. Average of the Inhibition Zone diameter for the two tested EOs against four microbes; *(√) completely inhibited microbial growth; (0) no microbial effect.*

Natural Oils		Inhibition Zone diameter (mm)			
		Aspergillus niger	*Penicillium cyclopium*	*Fusarium solani*	*Alcaligenes faecalis*
		Fungi			*Bacterium*
Thyme oil	5 %	8±0.00	11±0.30	0	3±0.00
	10%	11±0.29	14±1.1	√	7±0.11
	15 %	24±1.5	√	√	9±0. 13
Lemon oil	5 %	0	0	0	√
	10 %	8±0.19	9±0.11	√	√
	15 %	14±0.33	√	√	√
Negative Control (Ethanol 70%)		0	0	0	0

Data are the average of three replicates ± standard error

of the inhibition growth zone was 8mm for *Aspergillus niger* and 11 mm for *Penicillium cyclopium*. Thyme oil at 10% concentration completely inhibited the mycelia growth of *Fusarium Solani*, while the average diameter of the inhibition growth zone was 11 mm for *Aspergillus niger*, 14 mm for *Penicillium cyclopium*, and 7 mm for *Alcaligenes faecalis*. Thyme oil at 15% concentration completely inhibited the mycelia growth of *Penicillium cyclopium* and *Fusarium Solani*, while the average diameter of the inhibition growth zone of *Aspergillus niger* was 24 mm and 9 mm for the bacterium.

The powerful antibacterial activity of lemon oil in different concentrations was detected where all concentrations (5, 10, and 15 %) inhibited the growth of *Alcaligenes faecalis* completely. Lemon oil at 5% concentration has no antifungal effect against all the fungal strains, as there is no inhibition growth zone around the filter paper discs. Lemon oil at 10% concentration completely inhibited the mycelia growth of *Fusarium Solani*, while the average diameter of the inhibition growth zone was 8mm for *Aspergillus niger* and 9mm for *Penicillium cyclopium*. Lemon oil at 15% concentration completely inhibited the mycelia growth of *Penicillium cyclopium* and *Fusarium Solani*, while the average diameter of the inhibition growth zone of *Aspergillus niger* was 14mm. Ethanol 70% (as a negative control) has no antimicrobial effect against the four studied microbes.

3.5. Assessment for in vitro antimicrobial activity of EOs on replicas

Figures 12 and 13 illustrate the impact of antimicrobial activity of the two studied EOs oils against *Aspergillus niger*, *Penicillium cyclopium, Fusarium Solani,* and *Alcaligenes*

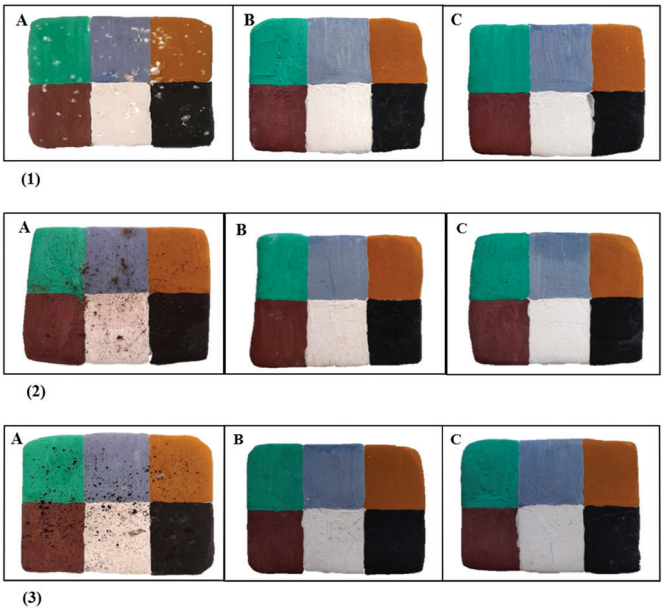

Figure 12. Antimicrobial activity of thyme EO against 1: Fusarium Solani + bacteriumon on replicas. 2: Penicillium cyclopium + bacterium on replicas. 3: Aspergillus niger + bacterium. (A) Control; (B) thyme EO 10 %; (C) thyme EO 15%.

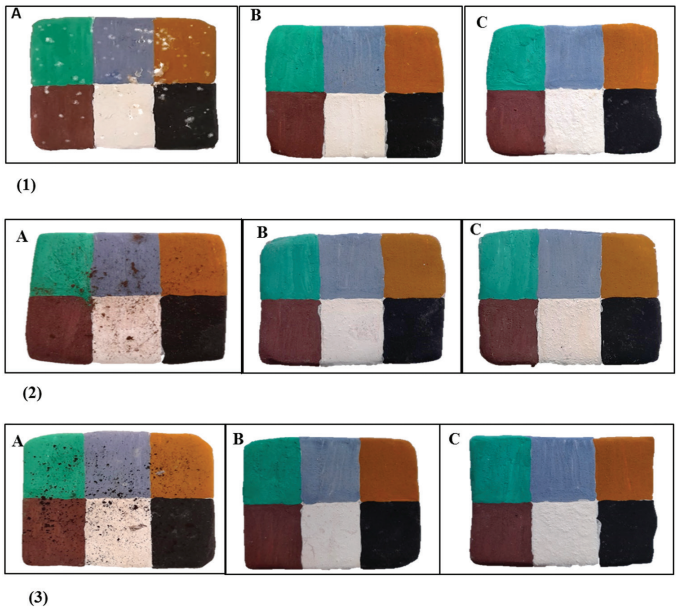

Figure 13. Antimicrobial activity of lemon EO against 1: Fusarium Solani + bacteriumon on replicas. 2: Penicillium cyclopium + bacterium on replicas. 3: Aspergillus niger + bacterium. (A) Control; (B) lemon EO 10 % ;(C) lemon EO 15%.

faecalis on agar plates proving the efficacy of the two essential oils at concentrations of 10% and 15%, so the two EOs at concentrations of 10% and 15% were applied to the replicas with the bacterium and the three studied fungi. After an incubation period of three months (considering they were examined every week) and by visual observation of the control replicas (only inoculated with bacterial and spore suspensions of the three tested fungi), it was found that a notable growth of fungi occurred on the surface of the replicas. The results of the visual examination of the other replicas (treated with EOs at different concentrations and inoculated with spore suspensions) revealed that the higher the oil concentration, the more efficient it is in inhibiting fungal growth; thyme oil is generally more efficient than lemon oil against the three studied fungi, thyme oil at 10% and 15% concentrations inhibited the growth of the three studied fungi on all the replicas (no fungal growth appeared on the surface of the replicas compared to the control replicas), while lemon oil inhibited the growth of the three studied fungi only at 15% concentration, while the 10% concentration only inhibited the growth of *Fusarium solani*.

By swabbing the different replicas to monitor bacterial abundance, it was noticed that the concentrations of lemon oil at 10 and 15 % have a powerful activity against *Alcaligenes faecalis,* as there is no visible growth by culturing the swabs on agar plates.

3.6. Colorimetric measurements

The obtained results of the colorimetric measurements of all pigments of the aged replicas treated with EOs at 10% and 15% concentrations were measured using a

spectrophotometer (the CIE L*,a*,b* color system was used). Total color change (ΔE) was calculated according to the following equation:

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

The results in Figure 14 show that no notable changes occurred due to the effect of the two EOs either in the concentration of 10% or 15%.

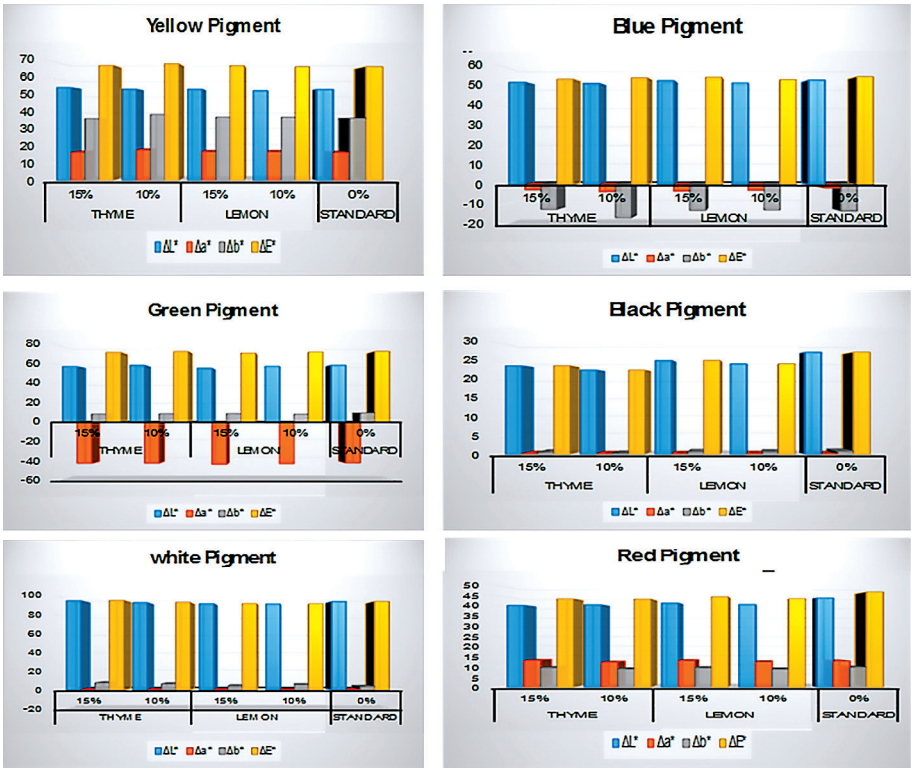

Figure 14. Change in color before and after treatment by EOs at 10% and 15% concentrations.

3.7. Chemical composition of EOs

The GC/MS analysis of the lemon oil showed a total of 49 volatile organic compounds, most of which are terpenoids; they were grouped into homologous series as follows: monoterpene hydrocarbons, oxygenated monoterpenes, oxygenated sesquiterpenes and sesquiterpenes, diterpenes. Table 2 shows that 17 compounds were classified as monoterpenes, with citral (15.34%), camphene, and 6-exohydroxy camphene (10.12%), and fenchone (7.14%) as the major compounds. Six compounds were listed as sesquiterpenes with funebrene (2.11%) as the major compound and only one compound that was listed as a diterpene, cembrene (0.55 %).

Table 2. Chemical composition of lemon oil analyzed by gas chromatography–mass spectrometry (GC-MS).

Monoterpene hydrocarbons	RT(min)	Peak area (%)	M.wt	M. formula
α-Pinene	7.57	0.13	136	$C_{10}H_{16}$
Camphene	7.65	0.57	136	$C_{10}H_{16}$
Delta 3-Carene	8.11	0.26	136	$C_{10}H_{16}$
α-Myrcene	9.93	5.13	136	$C_{10}H_{16}$
α-Terpinene	11.73	0.47	136	$C_{10}H_{16}$
γ-Terpinene	12.02	1.27	136	$C_{10}H_{16}$
α-Myrcene	22.73	0.11	136	$C_{10}H_{16}$
Oxygenated monoterpenes	RT(min)	Peak area (%)	M.wt	M.formula
Eucalyptol	11.17	1.03	154	$C_{10}H_{18}O$
Linalool oxide	12.46	0.16	170	$C_{10}H_{18}O_2$
6,6-dimethyl(CAS) beta-pinene oxide	13.12	0.18	152	$C_{10}H_{16}O$
Fenchone	13.39	7.14	152	$C_{10}H_{16}O$
Pulegone	14.43	0.86	152	$C_{10}H_{16}O$
α-Cyclocitral	15.35	3.09	152	$C_{10}H_{16}O$
Linalool	15.62	0.67	154	$C_{10}H_{16}O$
6-exohydroxy camphene	18.75	9.37	152	$C_{10}H_{16}O$
Citral	20.13	15.34	152	$C_{10}H_{16}O$
Sesquiterpenes	RT(min)	Peak area (%)	M.wt	M.formula
α-Funebrene	33.62	2.11	204	$C_{15}H_{24}$
Oxygenated Sesquiterpenes	RT(min)	Peak area (%)	M.wt	M.formula
Elemol	26.89	0.12	222	$C_{15}H_{26}O$
α-Cadinol	27.36	0.28	222	$C_{15}H_{26}O$
Farnesol (1st isomer)	32.46	0.11	222	$C_{15}H_{26}O$
Farnesol (2nd isomer)	35.79	0.19	222	$C_{15}H_{26}O$
Trans-Farnesol (3rd isomer)	36.41	0.21	222	$C_{15}H_{26}O$
Diterpenes	RT(min)	Peak area (%)	M.wt	M.formula
Cembrene	34.22	0.55	272	$C_{20}H_{32}$
Alkanes/alkenes, Aldhydes/ketones, Esters, ethers, and Orgnic acids	RT(min)	Peak area (%)	M.wt	M.formula
4-Cyclopropyl-4-penten1-ol	5.13	0.36	124	$C_8H_{12}O$
Salvene	6.86	0.12	124	C_9H_{16}
7,7-dichloro-6-[(methylsulfonyl)oxy-bicyclo-hept-2-ene	7.26	0.27	256	$C_8H_4D_6Cl_2O_3S$
7-Methylene-9-oxa-bicyclo-non-2-ene	9.49	1.47	136	$C_9H_{12}O$
6-Methyl-5-hepten-2-one	10.22	12.15	126	$C_8H_{14}O$
3,7,7-Trimethyl-1,3,5-cycloheptatri-ene	11.01	1.67	134	C_8H_{14}
2-methyl-2-hepten-5-yn4-one	12.89	0.36	122	$C_8H_{10}O$
Fenchyl acetate	13.97	0.23	196	$C_{12}H_{20}O_2$

(9-Oxabicyclo-non-6-en-3-yl) methanol	14.74	4.77	154	$C_9H_{14}O_2$
4,7,7-trimethylbicyclo-hept-4-ene-3-ol	15.98	1.12	152	$C_{10}H_{16}O$
3,5-Diacetoxy-2-methylcyclohexanone	16.25	0.11	228	$C_{11}H_{16}O_5$
2-Isopropenyl-1-methylcyclo-butane ethanol	17.39	0.42	154	$C_{10}H_{18}O$
3-cyano-5-hydroxy-2,5-dimethyl-4-oxy-pyrrole	18.81	8.52	152	$C_7H_8N_2O_2$
2-methoxy4,4,6-trimethyLcyclohexa-2,5-dien-1-one	19.63	4.36	166	$C_{10}H_{14}O_2$
1-[1-(trimethylsilyl)methyl-ethenyl] cyclo-pentanol	19.73	3.42	198	$C_{11}H_{22}OSi$
1,5,5-Trimethyl-6-methylene-cyclohexene	21.78	8.91	136	$C_{10}H_{16}$
1,4,4-Trimethylcyclohex-2-enyl)acetic acid	22.3	0.12	182	$C_{11}H_{18}O_2$
2-Oxo-1,2-dihydro-1-azaanthraquinone	23.29	0.7	225	$C_{13}H_7NO_3$
1-Heptatriacotanol	35.28	0.14	536	$C_{37}H_{76}O$
Hexadeca-2,6,10,14-tetraen-1-ol	35.96	0.15	290	$C_{20}H_{34}O$
Farnesol methyl ether	36.15	0.12	236	$C_{16}H_{28}O$
Methyl-5-oxo-2-phenyl-3-propylteTrahydrofuran-2-carboxylate	36.49	0.19	262	$C_{15}H_{18}O_4$
Oxiranecarboxaldehyde3-methyl-3- (4-methyl-3-pentenyl)	37	0.3	168	$C_{10}H_{16}O_2$
4-Propyl-3-oxabicyclo-decane	37.14	0.15	182	$C_{12}H_{22}O$
Linalyl isobutyrate	37.45	0.12	224	$C_{14}H_{24}O_2$

RT: retention time for each compound (minutes), peak area % represents the concentration of each compound in the oil sample, M. wt: molecular weight of the compound, and M. formula: molecular formula

Forty-six compounds for the thyme oil were reported, six compounds of monoterpene hydrocarbons were detected, of pinene (4.4%) and Camphene (2.03%), oxygenated monoterpenes of thymol (44.58%), linalool (3.28%) and cineole (1.64%), six compounds of sesquiterpenes were listed, and many other organic compounds that were classified as alcohols, esters, acids, and others (Table 3).

Table 3. Chemical composition of thyme oil analyzed by gas chromatography–mass spectrometry (GC-MS).

Monoterpene hydrocarbons	RT(min)	Peak area (%)	M.wt	M. formula
α-Thujene	7.88	0.46	136	$C_{10}H_{16}$
α-Pinene	8.03/8.12	4.40	136	$C_{10}H_{16}$
Camphene	8.55	2.03	136	$C_{10}H_{16}$
α-Terpinene	9.42	0.82	136	$C_{10}H_{16}$
Bornylene	10.47	0.58	136	$C_{10}H_{16}$
γ-Terpinene	12.13	0.60	136	$C_{10}H_{16}$
Oxygenated monoterpenes	RT(min)	Peak area (%)	M.wt	M.formula
1,8-Cineole	11.48	1.64	154	$C_{10}H_{18}O$

Borneol	15.70	0.68		154	$C_{10}H_{18}O$
L-Linalool	13. 70	3.28		154	$C_{10}H_{18}O$
Thymol	19.88	44.58		150	$C_{10}H_{14}O$
Sesquiterpenes	RT(min)	Peak area	(%)	M.wt	M.formula
α-Humulene	10.65/22.95	0.34/0.11		204	$C_{15}H_{24}$
α-farnesene	22.49	0.09		204	$C_{15}H_{24}$
γ-Cadinene	23.50	0.19		204	$C_{15}H_{24}$
Germacrene	24.41	0.19		204	$C_{15}H_{24}$
Cadinene	24.63	0.31		204	$C_{15}H_{24}$
α-Muurolene	27.31	0.28		204	$C_{15}H_{24}$
Oxygenated Sesquiterpenes	RT(min)	Peak area	(%)	M.wt	M.formula
Caryophyllenyl alcohol	25.71	0.08		222	$C_{15}H_{26}O$
Alkanes/alkenes, Aldhydes/ketones, Esters, ethers, and Orgnic acids	RT(min)	Peak area	(%)	M.wt	M.formula
Propanoic acid 2-methyl-tri-ethylsilyl ester	5.59	0.13		160	$C_7H_{16}O_2Si$
Krypton	6.62	0.10		84	Kr
2-Phenylacetic acid	7.96	1.40		136	$C_8H_8O_2$
2-Decen-1-ol	9.70	0.61		156	$C_{10}H_{20}O$
Geranyl propionate	10.02	3.29		210	$C_{13}H_{22}O_2$
1-(-1Hydroxy1methylethyl)-cyclohexanol	10.49	0.50		158	$C_9H_{18}O_2$
Benzenamine-,2,4,6-trimethyl	10.95	1.24		135	$C_9H_{13}N$
Dicinnamyl Ether	11.13	0.61		250	$C_{18}H_{18}O$
1-(Acetylmethyl)1-vinyl Cyclo-pentane	14.68	0.62		152	$C_{10}H16_O$
4-Fluoro-4-isopropylcyclo Hexa-2,5-dienone	14.94	0.44		154	$C_8H_7FO_2$
Endobornyl acetate	15.89	1.44		196	$C_{12}H_{20}O_2$
3-Cyclohexen-1-ol-4-methyl-1-(1-methylethyl)	15.96	0.27		154	$C_{10}H_{18}O$
3,7-Dimethylocta-2,6-dienyl 2-acetylbenzoate	16.39	1.16		300	$C_{19}H_{24}O_3$
1,3-Cyclohexadiene-1-methanol-1,2,6,6-tetramethyl	16.51	3.02		166	$C_{11}H_{18}O$
Methyl-4-(aminomethyl) benzoate	17.33	0.48		165	$C_9H_{11}NO_2$
Methyl thymyl ether	17.57	0.30		164	$C_{11}H_{16}O$
Estragole	18.89	2.15		148	$C_{10}H_{12}O$
4-nitrobenzoate	19.26	0.11		291	$C_{14}H_{10}ClNO_4$
2H(1)-4-MethoxyPhenyl-ethene	19.99	4.48		134	C_9H_9DO
Methyl Cinnamate	21.51	0.14		162	$C_{10}H_{10}O_2$
Methyleugenol	22.01	0.19		178	$C_{11}H_{14}O_2$
Benzyl (2-Methylpent-4-enyl) amine	22.24	2.54		189	$C_{13}H_{19}N$
Oxirane, (hexadecyloxy)methyl	31.37	0.16		298	$C_{19}H_{38}O_2$
2,5-Dimethyl-para-nisaldehyde	31.51	0.63		164	$C_{10}H_{12}O_2$

2,5-dimethyl anisole	32.62	0.26	136	$C_9H_{12}O$
2,4,5,6-Tetramethylpyrimidine	32.91	0.08	136	$C_8H_{12}N_2$
N-Cyanomethyl-2-azidobenzamide	34.03	0.09	201	$C_9H_7N_5O$
3-(4-Methyl-3-pentenyl)-3-cyclo-hexenyl pentylketone	34.42	0.20	262	$C_{18}H_{30}O$
Hexadecanoic acid,tri-methylsilyl ester	35.20	0.54	328	$C_{19}H_{40}O_2Si$

RT: retention time for each compound (minutes), peak area % represents the concentration of each compound in the oil sample, M. wt: molecular weight of the compound, and M. formula: molecular formula

3.8. Antimicrobial assay of thymol and citral

The antimicrobial activity results of 5, 10, and 15% of the two compounds are consistent with those obtained from the two EOs with slight changes (Table 4). For thymol, complete inhibition was observed only for *Penicillium cyclopium* at 10 and 15%. Citral at 15% concentration completely inhibited *Alcaligenes faecalis*, while the average diameter of the inhibition growth zones were 10mm, 11mm, and 12mm for *Fusarium Solani, Penicillium cyclopium, and Aspergillus niger* respectively. At 10% of citral, inhibition zones were detected only for *Aspergillus niger* and *Alcaligenes faecalis.* Thymol and citral at 5% concentration had no antifungal effect (there is no inhibition growth zone around the filter paper discs). Ethanol 70% (as a negative control) had no antimicrobial effect against the four studied microbes.

Table 4. Average diameter of the Inhibition Zone for the thymol and citral against four microbes, (√) completely inhibited microbial growth, (0) no microbial effect.

Chemical compound		Inhibition Zone diameter (mm)			
		Aspergillus niger	*Penicillium cyclopium*	*Fusarium solani*	*Alcaligenes faecalis*
		Fungi			*Bacterium*
Thymol	5 %	0	0	0	0
	10%	7±0.10	√	0	7±0.11
	15 %	18±0.14	√	12±0.48	8±0.66
Citral	5 %	0	0	0	12±0.21
	10 %	10±0.26	6	0	17±1.16
	15 %	12±0.09	11±0.9	10±0.81	√
Negative Control (Ethanol 70%)		0	0	0	0

Data are the average of three replicates ± standard errors

4. Discussion

In this study, the most predominant bacterium and three fungal species were isolated from the mural paintings of the Egyptian tomb of Khety (Tomb No. 17, 11th Dyn.). The mural paintings of the Khety tomb were executed using a tempra technique which is the main technique used in ancient Egypt [15]. Based on the swabs taken over the four seasons, the data indicates that the deterioration of the tomb was caused by the four predominant microbes *Aspergillus niger*, *Penicillium cyclopium, Fusarium Solani*, and *Alcaligenes faecalis*.

The results obtained regarding the antifungal activity of the two studied EOs (lemon and thyme oils) against *Aspergillus niger, Penicillium cyclopium, Fusarium Solani,* and *Alcaligenes faecalis* (the most common and dominant microbes in the tomb) on agar plates and replicas, revealed that all the studied EOs have variant inhibitory microbial effects and thyme oil at 15% concentration is generally more efficient than lemon oil against the three studied fungi at the same concentration. Some previous studies have also reported that lemon and thyme EOs have antifungal activity against many fungi isolated from archaeological objects [3].

Dimic et al. [16] tested the antifungal activity of lemon EO and coriander, and cinnamon extracts, by direct contact and vapour phase, indicating that the lemon essential oil showed complete inhibition of the growth of the tested microbes, while the cinnamon extract (in tested concentrations: 1.25-5.83 µl/ml) was the weakest inhibitor for the tested fungi.

The main difficulty in the dilution process of essential oils is their low water solubility, so the addition of solvents or detergents is unavoidable [17]. Most researchers have used ethanol or dimethyl sulfoxide (DMSO) to dilute essential oils [18,19]. Other researchers have used dimethylformamide, chloroform, and ethanol 70% to dilute essential oils; however, due to the damage/harm caused by dimethylsulfoxide (DMSO) and chloroform to archaeological materials and human health, they were excluded. The effectiveness of dimethylformamide (DMF), toluene, and ethanol 70% to dilute the studied EOs was first tested to determine the most effective solvent. The test revealed that ethanol 70% was the most effective solvent to dilute the studied EOs; it did not affect the color of the two tested EOs; it mixed very well with the oils; and when tested as a negative control it showed no antimicrobial effect against the three studied fungi. This result agreed with the results found by Gatti et al. [14].

The analysis of the volatile profile of the lemon oil shows that it mainly contains monoterpene, with citral, 6-exohydroxy camphene, and fenchone as the major compounds. These results conflict with many of the previously mentioned results [10] where limonene was the main constituent, with values of 78.93 and 53.57%, followed by b-pinene (5.08 and 7.44%) and supported by those of Tao et al. [20], who found that monoterpene hydrocarbons (C10H16) were the majority (88.96 percent, w/w) in *Citrus reticulata Blanco* essential oil, with citral, citronellol, octanal, linalool, decanal, nonanal, b-pinene, and c-terpinene as the major compounds. In our investigation, thymol was detected as the major compound in the thyme oil and this result is supported by those obtained by Shabnum and Wagay [21], who demonstrated that thymol and γ-terpinene were the main constituents of thyme oil. Satyal et al [22] also studied the composition of T. vulgaris oil from different regions, the oil sample from Jablanicki, Serbia, was of the geraniol chemotype (geraniol, 59.8%; geranyl acetate, 16.7%); the oil from Nyons, France, was of the linalool chemotype (linalool, 76.2%; linalyl acetate, 14.3%); and the essential oil from Richerenches, France, was of the thymol chemotype

(thymol, 47.1%; p-cymene, 20.1%), and the sample from Pomoravje District, Serbia, was of the sabinene hydrate chemotype (cis-sabinenehydrate, 30.8%; trans-sabinene hydrate, 5.0%).

It is quite difficult to compare the results obtained from different studies, due to the fact that the composition of the EOs can vary greatly depending on the geographical species and age of the plants, and methods of drying and extracting the oil. The mechanisms of the antimicrobial activity of EOs are not fully understood. Therefore, a plethora of studies suggest that this antifungal activity may be due to degrading of the cell wall, preventing the fungus to sporulate or the conidia to germinate, the flowing of cellular content, inactivating of extracellular enzymes, and thickening of the cytoplasm [23]. The antimicrobial activity of citral and thymol, and their concordance with the oils, suggest that the oil-forming compounds have a synergistic antimicrobial effect, which indicates that these compounds work together to eradicate microorganisms.

5. Conclusion

Lemon and thyme EOs diluted in ethanol 70% have antimicrobial activity against the most predominant microbes isolated from the mural paintings in the Egyptian tomb of Khety, both on agar plates and replicas. Results obtained for the two oils were slightly different, as thyme EO at 15% concentration was more effective than lemon oil against the three studied fungi. As regards antibacterial activity, lemon oil was the most effective. Colorimetric measurements showed that no notable changes occurred due to the treatment with the two tested EOs. Significant antimicrobial activity was observed using citral and thymol which were the major detectable compounds in the EOs.

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Biographical notes

Amany M. Reyad is currently a lecturer of microbiology at the Botany Department, Faculty of Science, Fayoum University. She received her doctorate degree in microbiology from Fayoum University in 2018. She has published several papers discussing different issues related to environmental and applied microbiology. She has taught many courses on the Microbiology Diploma course to students in the Faculty of Science, Fayoum, Egypt.

Mayssa Dayhoum Albasil is a lecturer in the conservation department at Luxor University, Egypt. She got her Ph.D degree in August 2021 in applied Microbiology in the conservation of mural painting from Fayoum University, Egypt. She got her Master degree in conservation of mural painting in 2015 from Cairo University, Egypt.

Gamal Mahgob is a professor in conservation of organic and inorganic materials in cultural heritage at Fayoum University, Egypt. He is a Vice Dean of the Faculty of Archeology, Fayoum University for education and students. He was formerly the Head of the Central Department of Conservation and Restoration at the Ministry of Antiquities. Professor Gamal Mahgoub is the supervisor of more than 100 master students and 93 Ph.D. students. He is an Editor for Scopus’ *Shedet* journal and a reviewer for 19 international journals. His main research interests are in applied nanotechnology in cultural heritage, laser technology in the conservation of culture heritage, and bio-treatment methodologies for archaeological monuments and sites.

Abeer ElHagrassy is an associated professor in conservation of inorganic materials and in applied microbiology in cultural heritage at Fayoum University Egypt. She is a head manager of the Research and Conservation Center, at the Faculty of Archaeology, Fayoum University. She is a scientific consultant for Takween and Megawra Company in Conservation Projects. She is a certified trainer at the Ministry of Antiquities, Egypt. She is a reviewer for Egyptian Biophysic journals and the international journal of microbiology. Her main research interests are in applied microbiology in environments, nanomaterial for the conservation of culture heritage, biodeterioration and biodegradation of monuments, bio treatment methods for archaeological monuments.

Summary

One bacterial species and five fungi were isolated from the mural paintings of the Egyptian tomb of Khety (Tomb No. 17, 11th Dyn.). The antimicrobial activity of lemon and thyme essential oils (EOs) diluted in ethanol 70% was evaluated against the bacterial isolate (related to the genus *Alcaligenes* and the species *faecalis* identified using 16 rRNA gene sequencing) and the most predominant fungi in the tomb, *Aspergillus ni-*

ger, Penicillium cyclopium and *Fusarium Solani* on agar plates using the disc diffusion method. Fragments were taken from the mural painting layers for analysis and examination to prepare replicas (5×5cm) similar to the archaeological mural painting and were artificially aged. Antimicrobial activity of the two studied EOs was evaluated against the bacterial isolate and the three studied fungi on replicas. Interestingly, our results revealed that the thyme oil at a concentration of 15% is generally more efficient than lemon EO against the three studied fungi and, in contrast, the lemon oil was more efficient against the bacterium than fungi. Moreover, colorimetric measurements proved that no notable changes occurred to the replica after treatment with the two tested EOs in 10% and 15%. From the GC-MS analysis, the volatile profile of the lemon oil revealed that it is mainly composed of monoterpenes, with citral, 6-exohydroxy camphene, and Fenchone as the major compounds. Our results showed that thymol (44.38%) is the major compound for the thyme oil.

Riassunto

Dai dipinti murali della tomba egizia di Khety (tomba n. 17, 11 din.) sono stati isolati una specie batterica e cinque funghi. L'attività antimicrobica degli oli essenziali di limone e timo (EO) diluiti in etanolo al 70% è stata valutata rispetto all'isolato batterico (relativo al genere Alcaligenes e alla specie faecalis identificato utilizzando il sequenziamento del gene 16 rRNA) e al fungo predominante nella tomba, Aspergillus niger, Penicillium cyclopium e Fusarium Solani su piastre di agar, utilizzando il metodo della diffusione del disco. Frammenti sono stati prelevati dagli strati della pittura murale per effettuare analisi su repliche (5×5 cm), simili alla pittura murale archeologica, invecchiati artificialmente. L'attività antimicrobica dei due EO studiati è stata valutata rispetto all'isolato batterico e ai tre funghi studiati sulle repliche. È interessante notare che risultati hanno rivelato che l'olio di timo, a una concentrazione del 15%, è generalmente più efficiente del limone contro i tre funghi studiati e, al contrario, l'olio di limone è più efficace contro il batterio rispetto ai funghi. Inoltre le indagini colorimetriche non hanno mostrato cambiamenti significativi nelle repliche dopo il trattamento con i due EO testati con concentrazione del 10% e del 15%. L'analisi GC-MS ha rivelato che il profilo volatile dell'olio di limone è composto principalmente da monoterpeni, con citrale, 6-esoidrossi canfene e Fenchone come composti principali. I risultati hanno mostrato che il timolo (44,38%) è il composto principale per l'olio di timo.