Bioactivities of Six Plant Essential Oils against some Isolated Microbes from an Archaeological Limestone Statue at the Saqqara Excavation

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The effect of microbial activity on the deterioration of archaeological stone items is a worldwide issue, and conserving them with low-toxicity, ecologically benign and naturally biocides is a difficult undertaking. Molecular identification of the microbial communities from a deteriorated archaeological object (Ptahshepses stone statue) located from the Saqqara excavation, Giza, Egypt was recorded. Six essential oils (EOs) (black cumin, clove, geranium, lavender, lemongrass, and thyme) were tested for antimicrobial activity against six degrading fungal and bacterial species. *Alternaria alternate*, *Aspergillus flavus*, *A. niger*, *Cladosporium halotolerans*, *Penicillium crustosum*, and *Trichoderma viride* and three bacterial species, *Pseudomonas protegens*, *P. putida*, and *Serratia odorifera*, were isolated. Of the most effective EOs, thyme showed the highest inhibition percentage (143.4%) against *Serratia odorifera*, followed by *P. putida* (135%), and *Pseudomonas protegens* (131.5%). Lemongrass and clove EOs had minimum inhibitory concentrations (MICs) ranging from 0.5 µL/mL to 2 µL/mL for all isolated deteriorated fungal and bacterial species, while the lowest efficiency EOs were lavender, geranium and black cumin. It can be concluded that thyme and lemongrass EOs have a potential use for protecting the Ptahshepses stone statue from microbial deterioration.

Keywords: Fungal species; Bacterial species; Antimicrobial activity; Essential oils; Microorganisms; Archaeological object; Excavation

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INTRODUCTION

Many studies deal with the treatment and conservation of the archaeological objects from the Saqqara excavation region (Afifi *et al.* 2020). Archaeological objects made from stone are an important part of the cultural heritage of the world. The history of humanness has been related to the use of stone, especially limestone, which was used for art objects, monuments, buildings, and private tombs, in which the walls were covered with paintings and reliefs in the ancient civilization in Egypt (Bard 2005). Ancient Egypt was considered

as the "stone state" because stone was the most utilized raw material during the several periods of Pharaonic Egypt (ElBaghdady *et al.* 2019).

The major biodeterioration agents of cultural heritage are fungal species, which are capable of colonizing, degrading, corroding, and changing a variety of materials that have been used through the centuries for the production of archaeological artifacts and monuments (Sterflinger 2010; Afifi 2012). Endolithic fungi live inside the pores and fissures of stone, while epilithic fungi grow on the rock, mortar, building stone, and plaster due to their higher activity (Scheerer *et al.* 2009; Sterflinger 2010). Several fungal species can cause staining of different colors and sizes, which are difficult to remove, as the fungal hyphae not only grows on the surface but also within the object (Strzelczyk 2004). For example, fungal deterioration aspects have been observed for an archaeological limestone relief panel in the Koom Oshem museum, El-Fayoum, Egypt, where *Alternaria alternate* was the most dominant species in the limestone relief followed by *Penicillium chrysogenum* (Afifi and Geweely 2006).

In addition, a majority of bacteria secrete some enzymes and secondary metabolite products to break down different organic substrates (Boer *et al.* 2005; Netzker *et al.* 2015; Zeilinger *et al.* 2016). ElBaghdady *et al.* (2019) stated that halotolerant bacteria (*Arthrobacter* spp., *Micrococcus* spp., *Bacillus* spp., and *Staphylococcus* spp.) were noticeable stone degrading isolates, while *Streptomyces* sp. was the primary agent causing color change in tomb paintings by producing different acids, *e.g.*, citric, oxalic, and succinic acid, as well as hydrogen sulfide and pigments. The chemical activity of biocides also affects treated materials (Agarossi *et al.* 1990). Gaseous fumigants can cause oxidization or polymerization, as well as corrosion of the treated materials (Daniels 1989; Soffritti *et al.* 2019). Chlorinated molecules can cause brittleness and discoloration of the treated ancient materials (Lee 2004). In addition, the thick and melanized cell walls of the fungi make them resistant to chemical attack and make them difficult to destroy with biocides or other antimicrobial treatments; black fungi settle deep within the granite, marble, and calcareous limestone, causing the stones to deteriorate (Sterflinger 2010).

Natural and environmentally-friendly alternatives, which show negligible toxicity to humans and the environment, have been used as improved antimicrobial agents for cultural heritage conservation (Marco *et al.* 2020). There has been considerable attention to using essential oils (EOs) as natural antimicrobial agents for the conservation of cultural heritage. The EOs and their components have been shown to have a broad-range of various properties including antibacterial (Lee *et al.* 2018; Okla *et al.* 2019; Behiry *et al.* 2020; Salem *et al.* 2021a), and antifungal (Veneranda *et al.* 2018; Hamad *et al.* 2019; Elgat *et al.* 2020; Mansour *et al.* 2020; Abd-Elkader *et al.* 2021; Ali *et al.* 2021; Mansour *et al.* 2021) properties.

EOs have shown potential antimicrobial activity against the isolated and identified microorganisms from archaeological objects (Mansour *et al.* 2020). *Pimpinella anisum*, *Origanum vulgare*, and *Allium sativum* EOs showed the best antifungal efficiency against four fungal strains, *i.e.*, *Aspergillus clavatus*, *A. niger*, *Penicillium* sp., and *Fusarium* sp., which were isolated from the Argentine and Cuban Documentary Heritage Museum; whereas, *Origanum vulgare* EO suppressed the mycelia growth and sporulation of *Aspergillus*, *Penicillium*, and *Fusarium* sp. (Borrego *et al.* 2012). In addition, a high antifungal activity was found for five EOs when tested on the Royal Tomb Paint in Tanis, Egypt (Sakr *et al.* 2012). Veneranda *et al.* (2018) investigated the antifungal efficacy of EO constituents, *i.e.*, thymol, eugenol, and cinnamaldehyde, on *A. niger* isolated from a Roman mural painting in Italy, to determine whether these constituents could be used for

middle and long-term protection applications. Recovery oil (α-bisabolol oxide A, d-limonene, and *α*-bisabolol oxide B), and EO (*Z-β*-farnesene, D-limonene, and *α*-bisabolol oxide A) obtained from fresh *Matricaria chamomilla* flowers showed potential antifungal activity against some molds isolated from archaeological objects, *e.g.*, *Aspergillus niger* (from archaeological manuscripts), *A. flavus* (from a museum gypsum board antique), *A. terreus* (museum archaeological tissue), and *F. culmorum* (museum organic materials) (El-Hefny *et al.* 2019).

The purpose of this research was to determine the efficacy of the lowest toxicity, environmentally-friendly, and naturally biocides in terms of the conservation of archaeological objects at a minimum effective dose. The isolation and molecular identification of deteriorating fungal and bacterial species from an archaeological object (Ptahshepses stone statue) from the Saqqara excavation, Giza, Egypt were recorded. An evaluation of the effect of six EOs (black cumin, clove, geranium, lavender, lemongrass, and thyme) on microbial growth was carried out. In addition, the EO treatments should not be hazardous to conservators or cause damage to the surfaces being treated.

EXPERIMENTAL

Investigation and Analysis of the Deteriorated Priest of Ptah Stone Statue

The statue was badly preserved in the stores of Saqqara, Giza Governorate, Egypt. The statue dates back to the old kingdom of the sixth dynasty, and is of a man seated on a chair, with a bas-relief in lines showing the name of the statue owner, read from left to right crosswise on the seat. The name is the priest of Ptah (Imbi). He is the holder of the royal sacraments, responsible for the establishment of justice for the god Maat. The statue suffered from many deterioration aspects, as follows: the face was broken from the whole statue; and it had been subjected to weathering effects from chemical, physical, and microbial actions. In addition, the Priest of Ptah statue suffered a loss in the painting layer with brown-black spots on its surface, which was accompanied by serious damage (Fig. 1).

X-Ray diffraction analysis (XRD)

The analysis and investigation studies were performed on the stone statue to identify the components of the statue, *i.e.*, the type of the stone and the deterioration products. At 40 kV and 30 mA, a Philips PW 3710/31 diffractometer with a -X target tube and Ni filter was used. The APD diffraction software from Philips was used to connect the Cu instrument to a computer system and the ASTM (American Society for Testing and Materials) card database for mineral identification.

Source of isolation

The Ptahshepses statue was found at the Saqqara excavation, Giza, Egypt. It was found in a bad state with many deterioration aspects. It had been subjected to weathering effects from chemical, physical, and microbial actions. The statue suffered a loss in the painting layer with brown-black spots on its surface accompanied by serious damage. The microbial species recorded in this research were isolated from the archaeological lime stone statue dated back to the old kingdom of the sixth dynasty. Multi-swabs were collected over several areas of the Ptahshepses stone statue and stored in its sterile packaging to identify the affecting fungal species (Fig. 1).

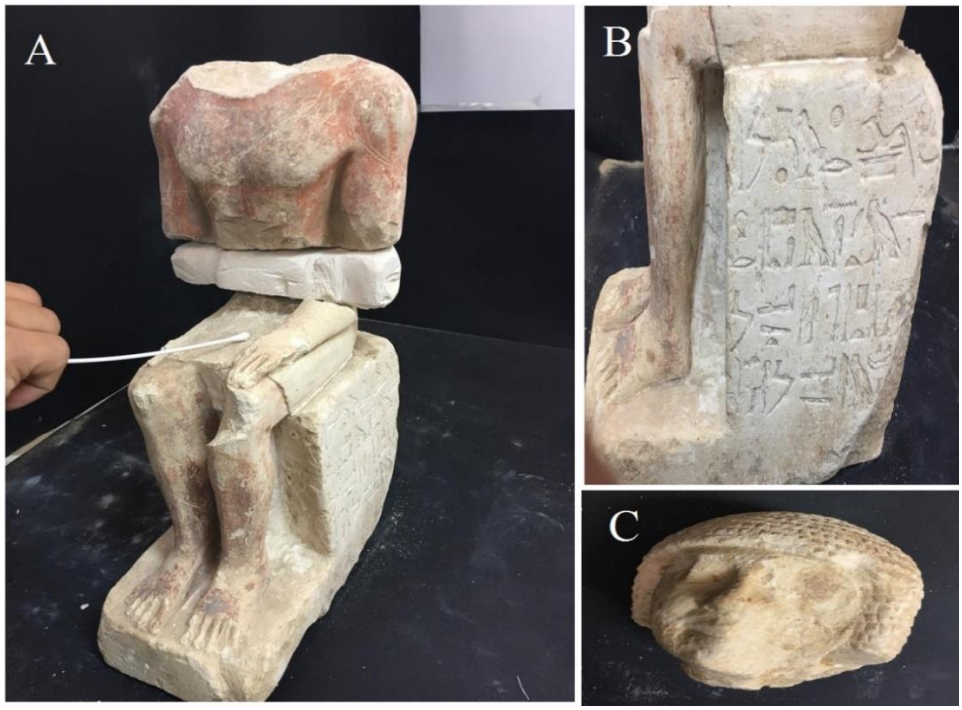

Fig. 1. The tested deteriorated Ptahshepses stone statue found at the Saqqara excavation, Giza, Egypt: (A) the whole stone statue without the face; (B) the loss in pigment layer; and (C) the broken face of the Ptahshepses stone statue

Isolation and identification of the deteriorating microbial species

The samples were obtained from the tested archaeological Ptahshepses stone statue discovered at the Saqqara excavation in Egypt. Sterile swabs were used on the damaged surface of the deteriorated archeological items. For fungi, the inoculated swabs were streaked across Czapeck's Dox agar dishes. These plates were incubated for 7 d at a temperature of 27 °C, while nutrient agar plates were incubated for 48 h at a temperature of 37 °C for the bacterial species (Corte *et al.* 2003). The isolated fungi were microscopically identified (Gilman 1957; Samson *et al.* 1981; Kern and Blevins 1997). The isolated bacterial colonies were purified, with every single purified colony identified according to Bergey's Manual of Systematic Bacteriology *via* biochemical tests (Cowan and Steel 1965; Cowan 2003; Vos *et al.* 2011). Total count and relative density of the isolated fungal species were measured accorded to Johnson *et al.* (1992) and Escudero and Mendoza (2005).

Molecular identification of the deteriorating fungal species

The sequences of the internal transcribed spacer (ITS) rDNA region were used to identify the isolated fungus species using molecular techniques. The DNA was extracted using the Quick DNA™ Fungal Microprep Kit (Sigma). The fungal universal primers ITS1 (50-TCCGTAGGTGAACCTGCGG-30) and ITS4 (50-TCCTCCGCTTATTGATATGC-30) were used to amplify the ribosomal DNA (White *et al.* 1990). Maxima Hot Start PCR Master Mix was used for PCR (polymerase chain reaction) (Thermo K1051). Electrophoresis of the PCR amplified products was performed in low melting agarose gels (1.5 percent) at 7 V/cm^2 for 1.5 h, and 0.5 g/mL of ethidium bromide (EtBr) was used to stain the PCR products, which were then observed under a 305 nm UV-light (Radford

1991). The PCR product was cleaned up using the Gene JET™ PCR Purification Kit (Thermo K0701). The GATC Company used an ABI 3730xl DNA sequencer to sequence the PCR product, employing forward and reverse primers with the latest 454 technology. The forward and reverse sequences of the DNA strand were aligned using the BLAST tool from the NCBI Gen Bank databases (National Center for Biotechnology Information).

Molecular identification of the deteriorating bacterial species

The bacterial species were grown in a nutrient broth for 24 h at a temperature of 30 °C, and then they were used for DNA extraction (Rashid *et al.* 2016). The DNA extraction was performed using a GeneJET™ Genomic DNA purification Kit (Thermo K0721) (Sigma) according to protocol outlined by the manufacturers (Riemann *et al.* 2000). A Maxima Hot Start PCR Master Mix (Thermo K1051) was used to carry out the PCR (Sigma). The PCR amplification was carried out using PCR with 50 µL of sample containing 25 µL of the Maxima Hot Start PCR Master Mix (2X), 5 µL of the template DNA, 18 µL of water (nuclease free), 1 µL (20 µM) of 27F forward primer, and 1 µL (20 µM) of 1492R primer. The 27F primer sequence was (5'-AGAGTTTGATCMTGG-CTCAG-3'), and the 1492R primer sequence was (5'-TACGGYTACCTTGTTACGACTT-3') (Lane). Amplification of the PCR products was performed using the following thermal cycling conditions: an initial denaturing step of 10 min at 95 °C (1 cycle), 35 cycles of denaturation at 95 °C for 30 s, annealing at 65 °C for 1 min, and elongation at 72 °C for 90 s, with a final extension cycle at 72 °C for 10 min.

A Gene JET™ PCR Purification Kit was used to clean up the PCR product (Thermo K0701). The GATC Company used an ABI 3730xl DNA sequencer to sequence the PCR product, employing forward and reverse primers with the latest 454 technology. The BLAST program (National Center for Biotechnology Information), available in the NCBI Gen Bank databases, was used to align the forward and reverse sequences of the DNA strand. The amplified 16S rDNA segment of the isolate was sequenced and compared to a number of non-redundant NCBI nucleotide sequence databases. The examination of hits from the mega-blast (very similar sequences) output was used to identify isolates. Sequences of 16S rDNA hits were used in a phylogenetic analysis of isolate 16S rDNA sequences in order to identify the evolutionary link between isolates and hits. The isolation 16S rRNA gene segments were sequenced and identified using standard techniques (Bhore *et al.* 2010).

Tested essential oils

Six essential oils (EOs) including black cumin (*Nigella sativa* seeds) clove (*Syzygium aromaticum* flower buds), geranium (*Pelargonium graveolens* leaves), lavender (*Lavandula angustifolia* leaves), lemongrass (*Cymbopogon citratus* leaves), and thyme (*Thymus vulgaris* leaves) were used. These EOs were provided by the Natural Oils Department, at the National Research Center in Dokki, Egypt. The EOs were extracted using a Clevenger-type device and hydrodistilled for 3 h (Badawy *et al.* 2017; Moustafa *et al.* 2021). Until needed, the extracted oils were kept in the dark at a temperature of 4 °C.

Antifungal activity of the tested essential oils

The antifungal activities of the six tested EOs were evaluated *via* the agar dilution method (Ishii 2017). The EOs were diluted in melted Czapeck's Dox agar at different concentrations (0.1, 0.25, 0.5, 1, and 2 µL/mL) with vigorous shaking, then poured in Petri plates (9 cm diameter) and allowed to solidify. Each plate was inoculated at the center with

a fungal disc (6 mm) from a 7-day-old culture. Itraconazole was used as a positive control, and sterilized distilled water was used as a negative control. All dishes were tested in triplicate and incubated at a temperature of 27 °C for 7 d. The inhibitory percentage of the tested EOs on the radial growth of the fungal mycelium was calculated according to Eq. 1,

$$Percent\ of\ inhibition = d_C - d_T \times \frac{100}{d_C} \quad (1)$$

where d_C is the average diameter (cm) of the fungal colony in the control and d_T is the average diameter (cm) of the fungal colony in a treatment group (Salem *et al.* 2021b).

Antibacterial activity of the six tested essential oils

The antibacterial activities of the six tested EOs were determined *via* the disc diffusion method, where the isolated bacterial species were swabbed on the surface of the nutrient agar in Petri dishes, then discs of Whatman filter paper (6 mm) were saturated with EOs at different concentrations (0.1, 0.25, 0.5, 1, and 2 µL/mL) and placed on the surface of the previously inoculated media. Chloramphenicol was used as the control. For 48 h, all the plates (in triplicate) were incubated at a temperature of 37 °C. The diameter of the inhibitory zone was measured in mm (Mosa *et al.* 2021). The percent inhibition (PI) was calculated for all EOs according to Eq. 2,

$$PI = \frac{Z_{oil}}{Z_s} \times 100 \quad (2)$$

where Z_{oil} is the mean zone of inhibition of each EO and Z_s is the zone of inhibition obtained for the standard antibiotic (Chloramphenicol) (Ali-Shtayeh *et al.* 2018).

Determination of the minimum inhibitory concentration (MIC) of the six tested essential oils on the isolated deteriorating fungal species

The agar dilution method was used to determine the minimum inhibitory concentration (MIC) of the six evaluated EOs. The tested EOs were diluted in melted Czapek´s dox agar at different concentrations (0.1, 0.25, 0.5, 1, and 2 µL/mL) with vigorous shaking, and then the media was poured into Petri dishes and let to solidify. Each plate was inoculated with a fungal disc (6 mm) in the center. The plates (in triplicate) were incubated at a temperature of 27 °C for 7 d. The MIC of the six tested Eos were expressed as the lowest concentration of EOs (µL of the EO/mL of culture medium) at which no visible growth occurred compared with the control (Adam *et al.* 1998)**.**

Determination of the minimum inhibitory concentration (MIC) of the six tested essential oils on the isolated deteriorating bacterial species

The MIC of the six tested EOs was demonstrated by diluting the EOs in nutrient agar at a temperature of 45 °C with different concentrations ((0.1, 0.25, 0.5, 1, and 2 µL/mL) with vigorous shaking. Then, the media was poured into Petri plates and allowed to solidify. Then the isolated bacteria was swabbed on the surface of the nutrient agar. The positive control consisted of the inoculated nutrient agar without EO. Non-inoculated plates containing essential oils were used as a negative control. Three plates (in triplicate) were incubated at a temperature of 37 °C for 48 h. The MIC of the tested EOs was expressed as the lowest concentration of EOs (µL of the EO/mL of culture medium) at which no visible growth occurred compared with the control (Oussalah *et al.* 2007).

Statistical Analysis

The antibacterial and antifungal activities of the EOs were calculated using the mean ± and standard deviation of the triplicate samples for each sample. The one-way ANOVA (analysis of variance) was done using Statistical Analysis Software (SAS, Release 8.02, Cary, NC, USA) system, which was followed by a Least Significant Difference (LSD) with a significant level of a *p*-value less than 0.05, according to Colao *et al.* (2005).

RESULTS AND DISCUSSION

X-Ray Diffraction Analysis (XRD)

The chemical composition of the statue, according to the results of the analysis, was essentially calcite ($CaCO_3$), the major component of limestone. However, traces of gypsum ($CaSO_4 \cdot 2H_2O$), quartz (SiO_2), halite (NaCl), and hematite (Fe_2O_3) were also detected (Fig. 2). According to Geweely *et al.* (2019), the primary components of archaeological wall painting stone is calcite, magnesium, and syn (Mg 0.06 Ca 0.94)(CO_3), which has a concentration of approximately 98%, while the high Ca, S, and O concentrations can be attributed to the ground painting layer matrix, which contains Si, C, and small amounts of Al, Mg, which may be interpreted as gypsum. Furthermore, the results supported the assertion by Clark and Zoitos (1992) that fungus grows best on quartz. Red ochre was documented to have been utilized in ancient Egypt (Casadio and Toniolo 2001).

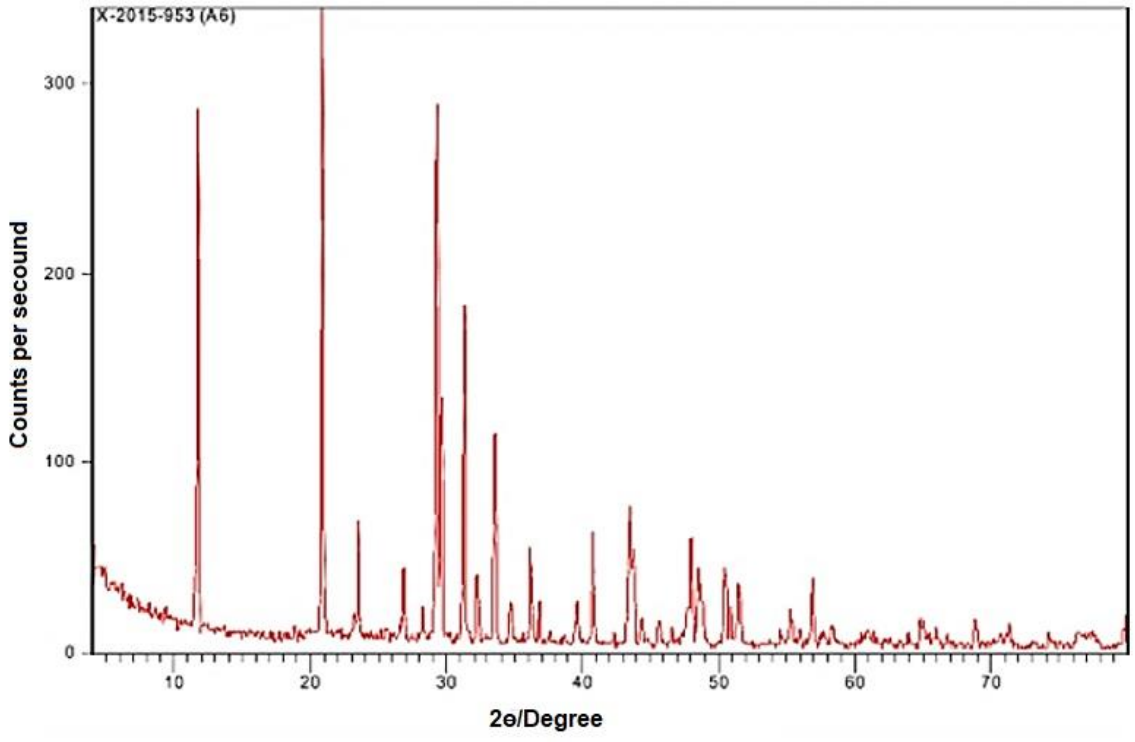

Fig. 2. The XRD pattern of the stone statue confirmed the presence of gypsum, calcite, hematite, halite and quartz

Isolation of the fungal and bacterial species

Six deteriorating fungal species (*Alternaria alternate*, *Aspergillus flavus*, *Aspergillus niger*, *Cladosporium halotolerans*, *Penicillium crustosum*, and *Trichoderma viride*), which accounted for 80 colonies, and three bacterial species (*Pseudomonas protegens*, *P. putida*, and *Serratia odorifera*) were isolated from the tested archaeological Ptahshepses stone statue found at the Saqqara excavation, Giza, Egypt (as shown in Table 1). The presence of fungal species on the tested stone statue for prolonged periods of time may be due to their potency at forming spores, which are more resistant to drought, as stated by Zhang *et al.* (2019). In addition, they stated that the fungal species are capable of producing different organic acids that deteriorate the stone surface, and also promote the development of other microorganisms. Furthermore, the fungal laccase systems are responsible for the resources competition and spreading on the stone surface.

Table 1. Total Count and Relative Density of the Fungal Species Isolated from the Tested Archaeological Ptahshepses Stone Statue Found at the Saqqara Excavation, Giza, Egypt.

Fungal Species	Count of Species	Relative Density (%)
Alternaria alternata	10	12.5
Aspergillus flavus	25	31.25
Aspergillus niger	19	23.75
Cladosporium halotolerans	3	3.75
Penicillium crustosum	16	20
Trichoderma viride	7	8.75
Total count	80	100
Number of species	6	

Aspergillus flavus and *A. niger* were the most dominant tested isolated fungal species with a relative density of 31.2% and 23.8%, respectively. The obtained results were in accordance with Geweely *et al.* (2019), who isolated *Aspergillus flavus* and *A. niger* from archaeological funeral masks in Saqqara, Egypt. The fungi had caused various deterioration aspects, *i.e.*, disintegration, discoloration, cracking, and stains. Mellon *et al.* (2007) stated that *A. flavus* has the ability to produce a broad range of hydrolytic enzymes that are substantial in terms of the nutrition and penetration of different organic substrates. *Aspergillus niger* is considered as a biodeterioration factor of culture heritage, as it can be adapted to various environmental conditions by different metabolic mechanisms (Zhang *et al.* 2017). The degradation action of *A. niger* is performed by the production of pectinases, hemicellulase, xylanases, and arabinases (Parenicová *et al.* 2000). *Aspergillus niger*, *Penicillium* sp., and *Cladosporium* sp. were the most frequent microorganisms in the deteriorated marble and stucco of the historic mosques in Cairo, Egypt (Afifi 2012; Aldosari *et al.* 2019). In addition, at the Saqqara Excavation in Egypt, researchers discovered a new comparative efficiency of ozone and gamma sterilization on the fungal deterioration of archeological painted coffins (Geweely *et al.* 2014).

The halophile microorganisms, *i.e.*, *Penicillium* sp. and *Aspergillus* sp., have developed various mechanisms of adaptation depending on the accumulation and biosynthesis of organic osmotic solutes, *i.e.*, sugar, sugar alcohols, amino acids, and amino acid by-products (Ortiz *et al.* 2014). The surfaces of the stone artifacts can be modified by hyphal penetration of fungi through the porous stone template and by the realization of the

pigments and organic acids (Sterflinger 2010; Kavita *et al.* 2011). Through oxidation, reduction, and transformation of metal ions, the acidification of the archaeological artefact enhances fungal growth and increases chemical biodeterioration (Gorbushina *et al.* 2004). *Penicillium* sp., *Alternaria* sp., *Allophoma* sp., and *Fusarium sp*. were isolated from the upper part of the Pompeian mural paintings conserved in the basement of the Ariadne House in Pompeii, Italy (Veneranda *et al.* 2017). *Pseudomonas* sp., *Bacillus* sp., *Alternaria alternate*, *Penicillium* sp., *Curvularia* sp., and *Fusarium* sp. caused deterioration of the archaeological walls of a pre-Hispanic ancient city in Mexico (Carrillo-González *et al.* 2016).

The most biodeteriorative microorganisms of cultural heritage were found to be fungal species *A. versicolor, Alternaria alternata, Aureobasidium* spp., *Chaetomium* spp., *Penicillium aurantiogriseum*, *P. chrysogenum*, *P. expansum*, *Stachybotrys chartarum*, *Trichoderma viride*, and *Ulocladium* spp., as well as bacterial species *Micrococcus*, *Aerobacter*, *Bacillus*, *Clostridium*, *Streptomyces*, *Staphylococcus*, and *Actinomyces* (Grabek-Lejko *et al.* 2017). In addition, the microorganisms responsible for the biodeterioration of the archeological objects in three Jordanian Museums (Yarmouk) are *A. niger, A. fumigates, A. nidulans, Penicillium chrysogenum, P. digitatum, P. italicum*, *Pythium* sp., yeast, *Bacillus subtilis*, *B. cereus*, *Micrococcus* spp., *Corynebacterium aquaticum*, *C. pseudodiphtheriticum*, *C. pyogenes*, *Escherichia coli*, *Listeria monocytogenes, Pseudomonas pseudoaclingenes*, *Salmonella* sp., and *Staphylococcus aureus* (Elserogy *et al.* 2016).

Alternaria, *Fusarium*, and *Penicillium* species were observed colonizing rocks (Sterflinger 2010). In addition, *Aspergillus*, *Beauveria*, *Curvularia*, *Cochliobolus*, *Chrysosporium*, *Conidiobolus*, *Fusarium*, *Penicillium*, *Sepedonium*, *Scopulariopsis*, *Trichothecium*, *Torula*, and *Ulocladium* genera were isolated from archaeological sites in India (Pandey *et al.* 2011). *Epicoccum nigrum*, *Alternaria alternate*, *A. atra*, *Dothiorella iberica*, and *Cladosporium cladosporioides* were isolated from archaeological stones in Fiesole (Italy) (Pinna *et al.* 2018).

The data from this study demonstrated that the three isolated bacterial species *(Pseudomonas protegens*, *P. putida*, and *Serratia odorifera*) were associated with the deterioration of the tested stone statue by different mechanisms (Zhang *et al.* 2019). These authors estimated that the bacteria produce color and pigments, as well as organic and inorganic acids, which are responsible for the destruction of the stone surface. Several bacterial species are able to grow in stone. For instance, *Micrococcus* was isolated from different types of stones, which produces exopolysaccharide substances (Urzì *et al.* 1991). Methanol, fructose methylamine, and trimethylamine are examples of products that halo-alkalophilic and methylotrophic bacteria can use as energy and carbon sources (Doronina *et al.* 1997, 2003). The level of decomposition will differ depending on the amount of organic material available (Caple 2001).

Molecular identification of the isolated fungal species

The six isolated fungal species, *i.e.*, *Alternaria alternate*, *Aspergillus flavus, A. niger*, *Cladosporium halotolerans*, *Penicillium crustosum*, and *Trichoderma viride*, from the tested archaeological Ptahshepses stone statue found at the Saqqara excavation were identified at the molecular level *via* the DNA sequencing of the PCR products. The six fungal species had the following accession numbers: *Alternaria alternate* (MN512215), *Aspergillus flavus* (MN517993), *A. niger* (MN513383), *C. halotolerans* (MN512648), *Penicillium crustosum* (MN512539), and *Trichoderma viride* (MN513046) (Table 2). The

phylogenetic trees showed their link with other related fungal species (Fig. S1 through Fig. S5).

Table 2. The Fungal and Bacterial Species Isolated from the Tested Archaeological Ptahshepses Stone Statue Found at the Saqqara Excavation and Their Accession Numbers

Class	Species	GenBank Accession Number
Fungal species	*Alternaria alternata*	MN512215
	Aspergillus flavus	MN517993
	A. niger	MN513383
	Cladosporium halotolerans	MN512648
	Penicillium crustosum	MN512539
	Trichoderma viride	MN513046
Bacterial species	*Pseudomonas protegens*	MN514045
	P. putida	MN514087
	Serratia odorifera	MN514046

Molecular identification of the isolated bacterial species

The three isolated bacterial species, *i.e.*, *Pseudomonas protegens*, *P. putida*, and *Serratia odorifera*, from the tested archaeological Ptahshepses stone statue found at the Saqqara excavation were identified at the molecular level *via* the 16S rRNA sequencing of the PCR products. The 16S rRNA gene of the three isolated bacterial species was amplified *via* PCR. The three bacterial species had the following accession numbers: *P. protegens* (MN514045), *P. putida* (MN514087), and *Serratia odorifera* (MN514046) (Table 2). The phylogenetic trees showed their link with other related bacterial species (Fig. S6 through Fig. S8).

The impact of the six essential oils on the radial growth of six isolated fungal species

The six EOs exhibited antifungal efficacy against six fungal species that were isolated from the Ptahshepses stone statue, found at the Saqqara excavation, Egypt (Table 3). *Thymus vulgaris*, *Syzygium aromaticum* and *Cymbopogon citratus* EOs were the most active. Based on the authors' previous works, the main compounds identified in the EO of lemongrass were α-citral (35.91%), *β*-citral (35%) 5-octyldihydro-2(3H)-furanone (9.08%), and nerylacetal (7.84%) (Moustafa *et al.* 2021). Eugenol was the abundant compound found in *S. aromaticum* EO (99.16%) (Salem *et al.* 2020). Thymol, *p*-cymene, and carvacrol were the most abundant compounds in *T. vulgaris* EO (Geweely *et al.* 2019). Among all the tested EOs, the thyme EO at a low concentration (0.5 µL/mL) exhibited the most efficient antifungal activity against all six isolated deteriorating fungal species, with the percent of inhibition ranging from 94.3% to 100%. Rota *et al.* (2008), reported that thymol and *p*-cymene were the major components of *T. vulgaris* EO, which had strong antimicrobial efficiency. The *p*-cymene compound is able to reduce ATP synthesis by decoupling the respiratory chain in microbial cells (Custódio *et al.* 2011). The breakdown of microbial cell membranes and the precipitation of cellular proteins is linked to the antibacterial activity of the phenolic chemicals in the thyme EO (ElBaghdady *et al.* 2019). In this relation, Noshyutta *et al.* (2016) stated that the fungicidal activity of the thyme EO can be used as an alternative preservative for an ancient Egyptian Coptic cellulosic manuscript.

Table 3. Antifungal Activity of the Six Essential Oils on the Six Isolated Deteriorating Fungal Species from the Archaeological Tested Ptahshepses Stone Statue *via* the Agar Dilution Method(*)

Essential Oils	Concentration	Inhibitory Percentage of Fungal Mycelium Radial Growth (%)					
		Alternaria alternata	*Aspergillus flavus*	*Aspergillus niger*	*Cladosporium halotolerans*	*Penicillium crustosum*	*Trichoderma viride*
Control#		$66.5^{k} \pm 0.2$	$69.2^{m} \pm 0.1$	$55.4^{j} \pm 0.2$	$27.8^{c} \pm 0.2$	$44.1^{g} \pm 0.1$	$76^{l} \pm 0.1$
Black cumin	0.1	$11.4^{b} \pm 0.2$	$7.8^{b} \pm 0.1$	$3.4^{a} \pm 0.0$	$13.7^{b} \pm 0.1$	$16.1^{b} \pm 0.1$	$22.8^{b} \pm 0.0$
	0.2	$23.3^{c} \pm 0.2$	$36.4^{f} \pm 0.3$	$21.4^{c} \pm 0.1$	$44.2^{f} \pm 0.1$	$32.1^{d} \pm 0.1$	$53.2^{g} \pm 0.1$
	0.5	$41.1^{f} \pm 0.2$	$63^{k} \pm 0.1$	$50^{h} \pm 0.0$	$72.2^{k} \pm 0.1$	$64.2^{j} \pm 0.2$	$75.8^{l} \pm 0.2$
	1	$58.5^{j} \pm 0.1$	$76.7^{o} \pm 0.1$	$60.3^{k} \pm 0.1$	$93.3^{o} \pm 0.1$	$84.1^{l} \pm 0.1$	$89.7^{o} \pm 0.2$
	2	$84.3^{p} \pm 0.1$	$87.5^{s} \pm 0.1$	$76.1^{r} \pm 0.1$	$100^{r} \pm 0$	$100^{o} \pm 0$	$100^{s} \pm 0$
Clove	0.1	$50.6^{i} \pm 0.3$	$42^{g} \pm 2.0$	$42^{f} \pm 0.1$	$32.5^{d} \pm 0.1$	$36.2^{e} \pm 0.1$	$43.1^{d} \pm 0.1$
	0.2	$68.5^{l} \pm 0.2$	$67.4^{l} \pm 0.2$	$65.9^{m} \pm 0.1$	$57.9^{h} \pm 0.1$	$84.1^{l} \pm 0.1$	$81.2^{m} \pm 0.1$
	0.5	$100^{t} \pm 0$	$86.2^{r} \pm 0.1$	$78.3^{s} \pm 0.1$	$81.3^{m} \pm 0.1$	$99.7^{o} \pm 0$	$97.3^{q} \pm 0.1$
	1	$100^{t} \pm 0$	$100^{u} \pm 0$	$99.3^{w} \pm 0$	$100^{r} \pm 0$	$100^{o} \pm 0$	$100^{s} \pm 0$
	2	$100^{t} \pm 0$	$100^{u} \pm 0$	$100^{w} \pm 0$	$100^{r} \pm 0$	$100^{o} \pm 0$	$100^{s} \pm 0$
Geranium	0.1	$43.2^{g} \pm 0.1$	$29.2^{d} \pm 0.1$	$38.4^{d} \pm 0.1$	$27.6^{c} \pm 0.2$	$28.1^{c} \pm 0.1$	$35.2^{c} \pm 0.1$
	0.2	$66.4^{k} \pm 0.2$	$43.2^{h} \pm 0.1$	$62.6^{l} \pm 0.1$	$51.2^{g} \pm 0.1$	$52.2^{h} \pm 0.2$	$54.3^{h} \pm 0.1$
	0.5	$86.7^{q} \pm 0.2$	$84.6^{q} \pm 0.3$	$71.6^{p} \pm 0.1$	$72.2^{k} \pm 0.2$	$76.2^{k} \pm 0.1$	$92.3^{p} \pm 0.2$
	1	$100^{t} \pm 0$	$99.67^{u} \pm 0$	$99.6^{w} \pm 0$	$99.3^{q} \pm 0$	$100^{o} \pm 0$	$100^{s} \pm 0$
	2	$100^{t} \pm 0$	$100^{u} \pm 0$	$100^{w} \pm 0$	$100^{r} \pm 0$	$100^{o} \pm 0$	$100^{s} \pm 0$
Lavender	0.1	$7.9^{a} \pm 0.1$	$1.5^{a} \pm 0.1$	$19.3^{b} \pm 0.1$	$9.1^{a} \pm 0.1$	$8^{a} \pm 0.0$	$10.1^{a} \pm 0.1$
	0.2	$25.2^{d} \pm 0.2$	$18.3^{c} \pm 0.1$	$39.8^{e} \pm 0.1$	$27.9^{c} \pm 0.1$	$32^{d} \pm 0.1$	$49.2^{e} \pm 0.1$
	0.5	$39.3^{e} \pm 0.2$	$52.2^{i} \pm 0.1$	$53.2^{i} \pm 0.1$	$44.2^{f} \pm 0.1$	$60^{i} \pm 0.0$	$68.2^{k} \pm 0.1$
	1	$49.2^{h} \pm 0.1$	$72.1^{n} \pm 0.1$	$67^{n} \pm 0.1$	$58.2^{h} \pm 0.1$	$76.3^{k} \pm 0.1$	$88.3^{n} \pm 0.2$
	2	$72.3^{m} \pm 0.1$	$86.2^{r} \pm 0.1$	$79.6^{t} \pm 0.2$	$74.2^{l} \pm 0.1$	$100^{o} \pm 0$	$100^{s} \pm 0$
Lemongrass	0.1	$58.8^{j} \pm 0.1$	$33.7^{e} \pm 0.1$	$53.4^{i} \pm 0.1$	46.3 ± 0.2	$40.2^{f} \pm 0.2$	$50.6^{f} \pm 0.1$
	0.2	$74.3^{n} \pm 0.1$	$55.2^{j} \pm 0.1$	$68.2^{o} \pm 0.1$	$65.2^{i} \pm 0.1$	$60.2^{i} \pm 0.1$	$67^{j} \pm 0.0$
	0.5	$88.3^{r} \pm 0.2$	$90.8^{t} \pm 0.1$	$87.4^{u} \pm 0.1$	$100^{r} \pm 0$	$88.2^{m} \pm 0.1$	$98.8^{r} \pm 0.1$

	1	$100^{t} \pm 0$	$100^{u} \pm 0$	$99.3^{w} \pm 0$	$100^{r} \pm 0$	$100^{o} \pm 0$	$100^{s} \pm 0$
	2	$100^{t} \pm 0$	$100^{u} \pm 0$	$100^{w} \pm 0$	$100^{r} \pm 0$	$100^{o} \pm 0$	$100^{s} \pm 0$
Thyme	0.1	$58.4^{j} \pm 0.2$	$55.2^{j} \pm 0.1$	$43.3^{g} \pm 0.2$	$39.3^{e} \pm 0.2$	$44.1^{g} \pm 0.1$	$63.1^{i} \pm 0.1$
	0.2	$78.2^{o} \pm 0.1$	$81.3^{p} \pm 0.1$	$73.8^{q} \pm 0.1$	$67.2^{j} \pm 0.1$	$96.4^{n} \pm 0.2$	$81.2^{m} \pm 0.1$
	0.5	$94.3^{s} \pm 0.2$	$100^{x} \pm 0$	$95.5^{v} \pm 0.1$	$95.3^{p} \pm 0.1$	$100^{o} \pm 0$	$100^{s} \pm 0$
	1	$100^{t} \pm 0$	$100^{u} \pm 0$	$99.6^{w} \pm 0$	$100^{r} \pm 0$	$100^{o} \pm 0$	$100^{s} \pm 0$
	2	$100^{t} \pm 0$	$100^{u} \pm 0$	$100^{w} \pm 0$	$100^{r} \pm 0$	$100^{o} \pm 0$	$100^{s} \pm 0$
LSD 0.05		5.93	6.32	5.71	7.98	6.34	5.48

Note: #: Positive control: Itraconazole
(*) is the percent inhibition of fungal growth; numbers are expressed as the mean ± standard deviation (n = 3) for each sample; and different letters in each column show mean value at a significant level (*p*-value less than 0.05). Means with the same letter/s within the same column are not significantly different according to LSD at level of probability 0.05.

The second highest ranked tested EO in terms of conservation of the tested archaeological object was the lemongrass EO. Lemongrass EO and its major components (citronellal and geraniol) have antimicrobial activity, which induces shrinkage of the cell wall and disruption of the cell membrane resulting in lysis of the microbial cells (Sahal *et al.* 2020). Citral and the other terpenes in lemongrass break down the integrity of the cellular wall to be disrupted, causing the homeostasis of the microbial cell to be disrupted (Hadjilouka *et al.* 2017). Therefore, exposure of the genes involved in fatty acid biosynthesis or peptidoglycan biosynthesis may affect the expression of lemongrass EO. In this relation, citral, which was extracted from the lemongrass EO, was tested against the *Aspergillus niger* collected from a mural painting in the basement of the Ariadne House, Pompeii, Italy (Veneranda *et al.* 2018). Lemongrass EO has antimicrobial efficiency against fungi, yeasts, and Gram-positive and Gram-negative bacteria (Mendes *et al.* 2020).

In the present study, the tested clove EO (1 µL/mL) showed significant inhibition against the six isolated fungal species with a percent of inhibition above 99%. The antifungal activity of clove EO may be assigned to the presence of eugenol. It was suggested that eugenol is an important element in the antifungal efficiency of clove EO (Xie *et al.* 2017). In addition, the findings of this study were consistent with the findings of Veneranda *et al.* (2018), who assessed the potential exploitation of clove EO as a novel preservative against the biological colonization of archaeological sites. The bioactive compound antifungal feature of the clove EO could be attributed to its lipophilic nature, which facilitated the penetration of the fungal membrane lipid bilayer and led to the disruption of the membrane (Sharma *et al.* 2018). The hydroxyl eugenol group can bind to proteins and block microbial amino acid decarboxylases in microbial species (Omonijo *et al.* 2018).

The tested geranium EO caused the complete inhibition of three isolated deteriorating fungal species, *i.e.*, *Alternaria alternata*, *Penicillium crustosum*, and *Trichoderma viride*, at 1 µL/mL, while the three rest of the fungal species were inhibited by 2 µL/mL of geranium. Citronellol and geraniol, as the major compounds of geranium EO, as noted by Zore *et al.* (2011), can damage membrane integrity, impedes germs, and inhibits the microbial cell cycle, which may be due for the antimycotic activity of the test geranium EOs. The tested black cumin EO showed moderate activity against the isolated fungal species at low concentrations, and its activity increased as the EO concentration increased. The antimicrobial efficiency of the tested black cumin EO may be related to the presence of a high level of phenolics in the black cumin EO, which are considered as powerful active compounds with a strong antimicrobial efficiency (Luther *et al.* 2007). The black cumin EO contained high concentrations of thymoquinone, and its associated compounds, *i.e.*, thymol and dithymoquinone, which have antimicrobial activities (Singh *et al.* 2005). The components of the black cumin EO are directly related to the inhibition of fungal cell growth, which depends on the used dose (Santos *et al.* 2018).

The least efficient EO was the lavender EO, which had the lowest efficient antifungal activity against all six isolated deteriorating fungal species at the highest concentration (2 µL/mL) with the percent of inhibition ranging from 72.3% to 100%. Rakotonirainy and Lavédrine (2005) suggested that the antifungal properties of *Lavandula angustifolia* EO against fungal species isolated from the library and archive storage regions were due to the presence of linalool and linalool acetate as the major components.

Aspergillus niger was the most resistant species to the six tested essential oils, which may due to several resistance mechanisms, *i.e.*, producing biofilms, permeability of cell walls, over-expression of the target site, modifications to paths, incompatibility with

the objective, system of drug efflux, and reaction to stress, as recorded by Roilides and Iosifidis (2019). These authors stated that the resistance of fungal species, including *Aspergillus* spp., against antifungal drugs may be due to primary resistance or secondary mechanisms of resistance after exposure to antifungals and the selection of resistant clones.

The impact of the six tested essential oils on the growth of the isolated bacterial species of the stone statue

The results in Table 4 reveal that the six tested EOs at different concentrations (0.1, 0.25, 0.5, 1, and 2 µL/mL) exhibited a variable degree of antibacterial activity against the three isolated deteriorating bacterial species.

Table 4. Antibacterial Activity of the Six Essential Oils on the Three Isolated Deteriorating Bacterial Species from the Archaeological Tested Ptahshepses Stone Statue *via* the Disc Diffusion Method (*)

Essential Oils	Concentration (µL/mL)	Percent of Inhibition (PI%)		
		P. protegens	*P. putida*	*S. odorifera*
Control		$15.7^{d} \pm 0.3$	$20^{c} \pm 0.6$	$29.7^{d} \pm 0.3$
Black cumin	0.1	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$
	0.2	$12.5^{c} \pm 0.1$	$9.7^{b} \pm 0.3$	$33.1^{e} \pm 0.1$
	0.5	$50.3^{i} \pm 0.1$	$30^{e} \pm 0.1$	$62.8^{j} \pm 0.4$
	1	$68.5^{k} \pm 0.1$	$64.2^{j} \pm 0.4$	$85.9^{o} \pm 0.5$
	2	$93.8^{m} \pm 0.0$	$80.3^{lm} \pm 0.9$	$95.9^{q} \pm 0.5$
Clove	0.1	$25.2^{f} \pm 0.1$	$43.7^{g} \pm 1.3$	$29.3^{d} \pm 0.7$
	0.2	$62.4^{j} \pm 0.1$	$79.7^{l} \pm 0.9$	$36.6^{f} \pm 0.3$
	0.5	$81.2^{l} \pm 0.1$	$100^{o} \pm 0.0$	$65.6^{k} \pm 0.8$
	1	$100^{n} \pm 0.0$	$105.8^{p} \pm 1.0$	$92.8^{p} \pm 0.4$
	2	$111.2^{q} \pm 0.7$	$116.5^{r} \pm 0.8$	$103.4^{s} \pm 0.3$
Geranium	0.1	$18.6^{e} \pm 0.1$	$24.2^{d} \pm 0.6$	$20.3^{c} \pm 0.9$
	0.2	$43.9^{h} \pm 0.1$	$55.3^{h} \pm 0.3$	$40^{g} \pm 0.6$
	0.5	$81.2^{l} \pm 0.1$	$70.3^{k} \pm 0.9$	$62.8^{j} \pm 0.4$
	1	$100^{n} \pm 0.0$	$100^{o} \pm 0.0$	$79.7^{n} \pm 0.3$
	2	$107^{o} \pm 0.5$	$114.7^{r} \pm 0.3$	$100^{r} \pm 0.0$
Lavender	0.1	$6.2^{b} \pm 0.1$	$0^{a} \pm 0.0$	$0^{a} \pm 0.0$
	0.2	$18.7^{e} \pm 0.1$	$19.7^{c} \pm 0.3$	$16.6^{b} \pm 0.3$
	0.5	$43.7^{h} \pm 0.1$	$60.7^{i} \pm 1.2$	$29.7^{d} \pm 0.3$
	1	$62.2^{j} \pm 0.2$	$82^{m} \pm 1.2$	$72.4^{m} \pm 0.7$
	2	$100^{n} \pm 0.0$	$100^{o} \pm 0.0$	$100^{r} \pm 0.0$
Lemongrass	0.1	$37.5^{g} \pm 0.1$	$33.7^{f} \pm 0.9$	$35.9^{f} \pm 0.5$
	0.2	$62.3^{j} \pm 0.1$	$64^{j} \pm 0.6$	$52.8^{h} \pm 0.4$
	0.5	$100^{n} \pm 0.0$	$95^{n} \pm 0.6$	$86.6^{o} \pm 0.3$
	1	$112.6^{r} \pm 0.4$	$110.8^{q} \pm 0.4$	$105.4^{t} \pm 0.4$
	2	$118.3^{s} \pm 0.6$	$129.2^{t} \pm 0.6$	$116.6^{u} \pm 0.3$
Thyme	0.1	$37.4^{g} \pm 0.1$	$61^{i} \pm 1.0$	$56.1^{i} \pm 0.5$
	0.2	$68.9^{k} \pm 0.1$	$81^{lm} \pm 0.6$	$70.5^{l} \pm 0.3$
	0.5	$93.9^{m} \pm 0.1$	$100^{o} \pm 0.0$	$100^{r} \pm 0.0$
	1	$109.5^{p} \pm 0.3$	$122.5^{s} \pm 0.3$	$120.5^{v} \pm 0.3$
	2	$131.5^{t} \pm 0.3$	$135^{u} \pm 0.5$	$143.4^{w} \pm 0.3$
LSD 0.05		8.02	8.28	7.81

Note: (*) is the percent inhibition of bacterial growth; numbers are expressed as the mean ± standard deviation (n = 3) for each sample; and different letters in each column show mean value at a significant level (*p*-value less than 0.05). Means with the same letter/s within the same column are not significantly different according to LSD at level of probability 0.05.

The tested thyme and lemongrass EOs were the most effective significant EOs against the three isolated bacterial species. At the highest concentration (2 µL/mL), the tested thyme EO showed the highest inhibition percentage (143.4%) against *Serratia odorifera*, followed by *P. putida* (135%), and *Pseudomonas protegens* (131.5%). This result may be due to the presence of thymol, which causes bacterial cell wall damage, as well as carvacrol and thymol sensitizing microbial cell walls, which results in cell wall degradation, cytoplasmic membrane damage, cytoplasm coagulation, membrane protein destruction, proton motive force decrease, and microbial cell dissolution (Nazzaro *et al.* 2013). Thyme EO has antibacterial activity at very low concentrations (0.5%) against almost all rock colonizing bacteria isolated from different archaeological limestone objects, in Egypt (ElBaghdady *et al.* 2019). The *Thymus* EO inhibited the growth of *Bacillus subtilis* from an ancient Egyptian Coptic cellulosic manuscript at a concentration of 0.1% v/v (Noshyutta *et al.* 2016).

The tested lemongrass EO, at a concentration of 2 µL/mL, exhibited significant inhibition of the three isolated deteriorating bacterial species, ranging from 116.6% to 129.2%. The obtained results agreed with Mehmood *et al.* (2018), who found that lemongrass EO has inhibitory activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli*. The phenolic compounds in lemongrass EO might be responsible for the antimicrobial activity by changing the permeability of the cell wall, reacting with cell membrane phospholipids, and denaturing the proteins (Klangmuang and Sothornvit 2016). Lemongrass EO penetrates the bacterial cytoplasmic membrane, causing intracellular component leakage and, as a result, cell death (Mishra *et al.* 2018). The major components of lemongrass EO, *i.e.*, citronellal, citronellol, nerol, and geraniol, increase the membrane fluidity and permeability of the microorganisms, induce cellular lysis, and damage the cytoplasmic membrane integrity of these microorganisms (Cunha *et al.* 2020).

Moderate antibacterial activity was found in the clove and geranium EOs. It was found that the clove EO damaged the *Bacillus* sp. membrane, which were taken from the National Archive of the Republic of Cuba and the Historical Archive of the Museum of La Plata in Argentina (Borrego *et al.* 2012). The hydrophobicity of the clove EO and its components enabled them to distribute into the lipids of the bacterial cell membrane and mitochondria, interfering with these structures. This caused these structures to become more permeable, which affects cell metabolism and finally causes bacterial death (Burt 2004). Citronellol, citronellyl formate, and geraniol are the primary ingredients of geranium EO, and they have an antibacterial impact against Gram-positive bacteria more than Gram-negative bacteria (Boukhatem *et al.* 2013).

The least efficient essential oils were lavender and black cumin. The obtained data agreed with Yuan *et al.* (2019), who showed that lavender oil has antifungal, antibacterial and antioxidant properties. The black cumin EO has antimicrobial activity against Gram-negative bacteria due to their active compounds, *i.e.*, carvone, D-limonene, α-pinene, and *p*-cymene (Konuk Takma and Korel 2019).

It was found that there was no difference in the diameter of the inhibition zone against of *Bacillus subtilis* at all concentrations of *Lavandula angustifolia* (5 mg/mL, 10 mg/mL, and 20 mg/mL), where the diameter of the inhibition zone of *B. subtilis* at the highest concentration (20 mg/mL) was 13.43 mm (Rashed *et al.* 2017). Lavender EO showed moderate antibacterial activity, while black cumin oil did not show any inhibitory effect against any of the tested bacterial species (Al-Nabulsi *et al.* 2020). It was suggested that the redox potential action of black cumin EO could aid in the adsorption and neutralization of free radicals as well as the chelation of metals (Bettaieb *et al.* 2010).

The MIC values of the different tested EOs are shown in Table 5. The MICs of the thyme and lemongrass EOs ranged from 0.5 to 2 µL/mL for all the tested deteriorating fungal species, while the MIC values for the three isolated bacterial species ranged from 0.5 to 1 µL/mL. The obtained data agreed with Boubaker *et al.* (2016), who indicated that the *Thymus* EO showed complete inhibition (100%) of spore germination for *Penicillium* sp. at a concentration of 0.5 µL/mL. Thyme EO at a concentration of 0.5 µL/mL had a strong inhibitory activity on the growth of *Aspergillus* sp. (Božik *et al.* 2017). Lemongrass EO has strong antimicrobial activity with a MIC value of 0.025% for *Staphylococcus aureus* and 0.5% for *Escherichia coli* (Mendes *et al.* 2020).

Table 5. Minimum Inhibitory Concentration (MIC) of Six Tested Essential Oils (Black Cumin, Clove, Geranium, Lavender, Lemongrass, and Thyme) on the Isolated Microbial Species from the Ptahshepses Stone Statue from the Saqqara Excavation, Giza, Egypt

Microbial Species	MIC of the Essential Oils (µL/mL)					
	Black cumin	Clove	Geranium	Lavender	Lemongrass	Thyme
A. alternata	> 2*	0.5	1	> 2	1	1
A. flavus	> 2	1	2	> 2	1	0.5
A. niger	> 2	2	2	> 2	2	2
C. halotolerans	2	1	2	> 2	0.5	1
P. crustosum	2	1	1	2	1	0.5
T. viride	2	1	1	2	1	0.5
P. protegens	> 2	1	1	2	0.5	1
P. putida	> 2	0.5	1	2	1	0.5
S. odorifera	2	2	2	2	1	0.5
Note: * the MIC of the six tested essential oils expressed as the lowest concentration of essential oils (µL of the essential oil per mL of culture medium) at which no visible growth was occurred compared with control						

The MIC of the clove EO ranged from 0.5 to 2 µL/mL for all the isolated deteriorating fungal and bacterial species, while the MIC of the geranium EO ranged from 1 to 2 µL/mL for all the isolated deteriorating fungal and bacterial species. The thyme and clove EOs were highly active at a concentration of 0.75 to 1.5 mg/mL against *P. fluorescens, E. coli*, *Serratia marcescens*, *S. aureus*, *Micrococcus* spp., *Sarcina* spp., and *Bacillus subtilis* (Farag *et al.* 1989). The obtained results agreed with Sienkiewicz *et al.* (2014), who showed that geranium EO had the greatest antibacterial efficiency against various bacterial species with a MIC ranging from 3.0 to 8.0 µL/mL.

The MIC of the tested lavender EO was 2 µL/mL for *Penicillium crustosum*, *Trichoderma viride, P. protegens*, *P. putida*, and *Serratia odorifera*, while the MIC for *Alternaria alternate, A. flavus, A. niger*, and *Cladosporium halotolerans* was greater than 2 µL/mL. The MIC of the black cumin EO was 2 µL/mL for *C. halotolerans, Penicillium crustosum, Trichoderma viride,* and *S. odorifera.* The inhibitory effect of the black cumin EO against some pathogenic bacterial species had an MIC ranging from 100 to 200 µg/mL

(Hassanien *et al.* 2014).

When thymol collaborated with the cell membranes, it affected membrane permeability, which led to a lack of membrane potential with outflows of adenosine 5′-triphosphate (ATP), carboxyfluorescein, and potassium ions (Hyldgaard *et al.* 2012). Thymol also affected many enzymes directly or indirectly that were involved in the synthesis of ATP and destroyed the citrate metabolic pathway (Pasqua *et al.* 2010). The potency of the cells to improve after exposure to thymol is very low because thymol affects critical energy-generating processes. Thymol damages ergosterol biosynthesis as well as disrupts vesicles and cell membranes in *Candida* strains because ergosterol regulates membrane asymmetry and fluidity (Hyldgaard *et al.* 2012). Additionally, thymol showed considerable fungicidal efficiency against *A. flavus* through the reactive oxygen species scavengers.

Found in thyme EO, *p*-cymene is a monoterpene that has an inadequate antimicrobial efficiency when used alone (Bagamboula *et al.* 2004). However, it can induce the efficiency of some compounds, *e.g.*, polymyxin B nonapeptide and carvacrol (Rattanachaikunsopon and Phumkhachorn 2010). Several studies have shown that *p*-cymene can act as an alternative impurity in the microbial membrane, which partly disturbs it. As such, *p*-cymene causes the expansion of the membrane and influences the membrane potential of undamaged cells and causes a reduction in the enthalpy and melting temperature of membranes (Cristani *et al.* 2007). In addition, *p*-cymene can affect protein synthesis and cell motility (Burt Sara *et al.* 2007).

The lemongrass EO and its citral components are capable of antifungal activity against *A. niger*, *Alternaria alternate*, *Fusarium moniliforme*, and *F. solani via* damaging their cell wall and cell membrane to a noticeable degree. In addition, they can cause partial cytoplasm leakage by inhibiting DNA, RNA, protein, and biosynthesis of peptidoglycan as well as interfering with the ergosterol biosynthesis in *Candida albicans* (Nazzaro *et al.* 2013).

Eugenol is the fundamental ingredient in clove EO, and its antimicrobial efficiency is related to its ability to interact with proteins and permeabilize the cell membrane (Goñi *et al.* 2016). Eugenol affects the membranes *via* the non-distinct permeabilization of the cytoplasmic membrane, which increases the transport of ATP and potassium out of the cells (Hyldgaard *et al.* 2012). Eugenol causes alterations in the fatty acid profile of *Brochotrix thermosphacta*, *E. coli*, *Salmonella enterica*, and *S. aureus* (Di Pasqua *et al.* 2010).

An inhibitory effect was found for EOs relative to the growth and physiological activity of the deteriorating fungal species isolated from three archeological objects found at the Saqqara excavation, Egypt (Geweely *et al.* 2020). In addition, the antimicrobial activity of gold nanoparticles (AuNPs) in terms of the deterioration of archeological gilded painted cartonnage, from the late period, in Saqqara, Egypt was tested (Afifi *et al.* 2016).

In conclusion, these essential oils are recommended as they are not toxic to the conservators and cannot cause damage to the surfaces treated.

CONCLUSIONS

1. Chemical and mechanical preservation are not effective agents for eliminating deteriorating microbial species. Therefore, the primary recommendation of this research is the usage of natural and environmentally-friendly treatments for the

preservation of stone archaeological objects.

2. The development of natural biocides helps reduce the negative impact of synthetic agents as well as being effective, selective, biodegradable, and less toxic to the environment.
3. Most essential oils have some degree of antimicrobial activity attributable to the presence of terpenoid and phenolic compounds.
4. The obtained results confirmed that the thyme and lemongrass oils were potentially useful for protecting the Ptahshepses stone statue found at the Saqqara excavation, in Giza, Egypt from microbial deterioration.

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APPENDIX

Supplementary Materials

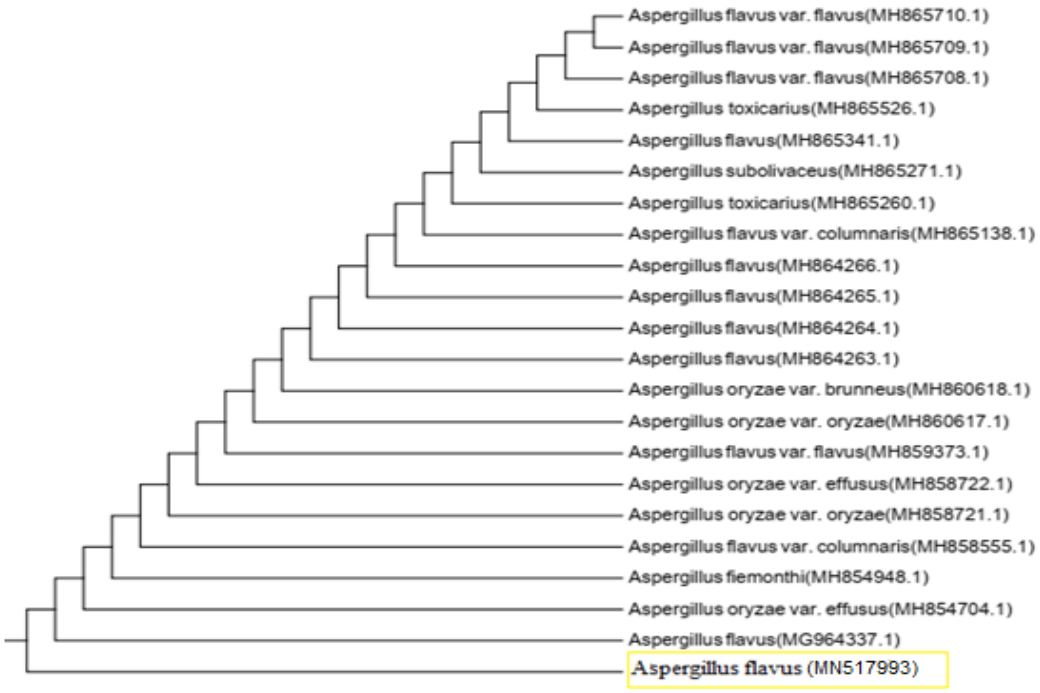

Fig. S1. Phylogenetic analysis tree of *Aspergillus flavus* (MN517993)

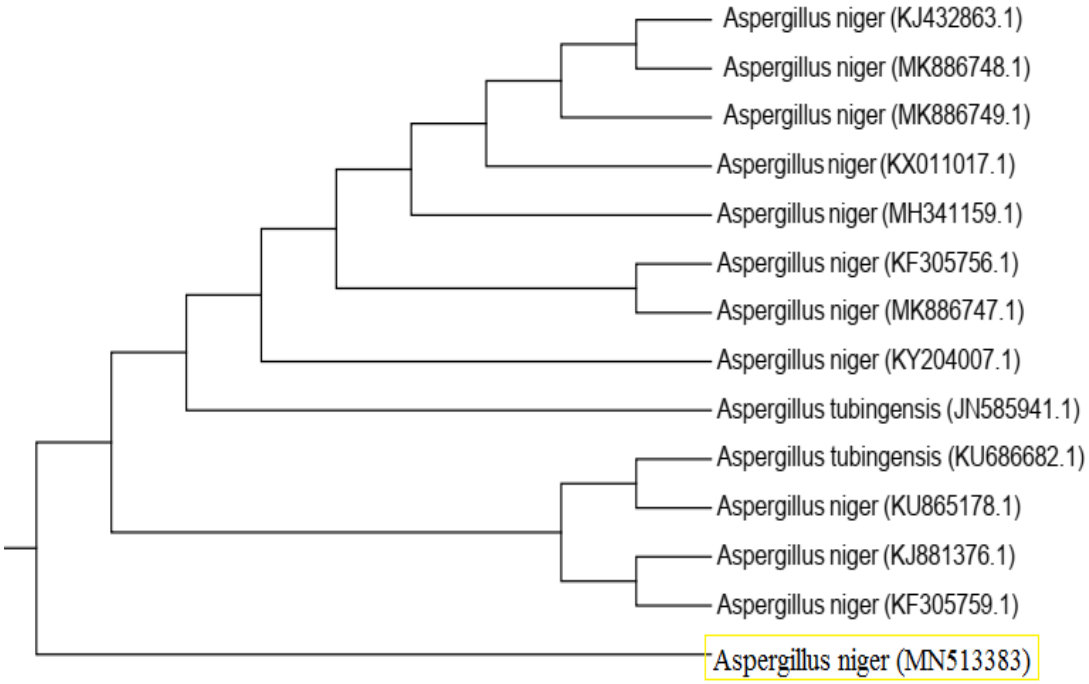

Fig. S2. Phylogenetic analysis tree of *Aspergillus niger* (MN513383)

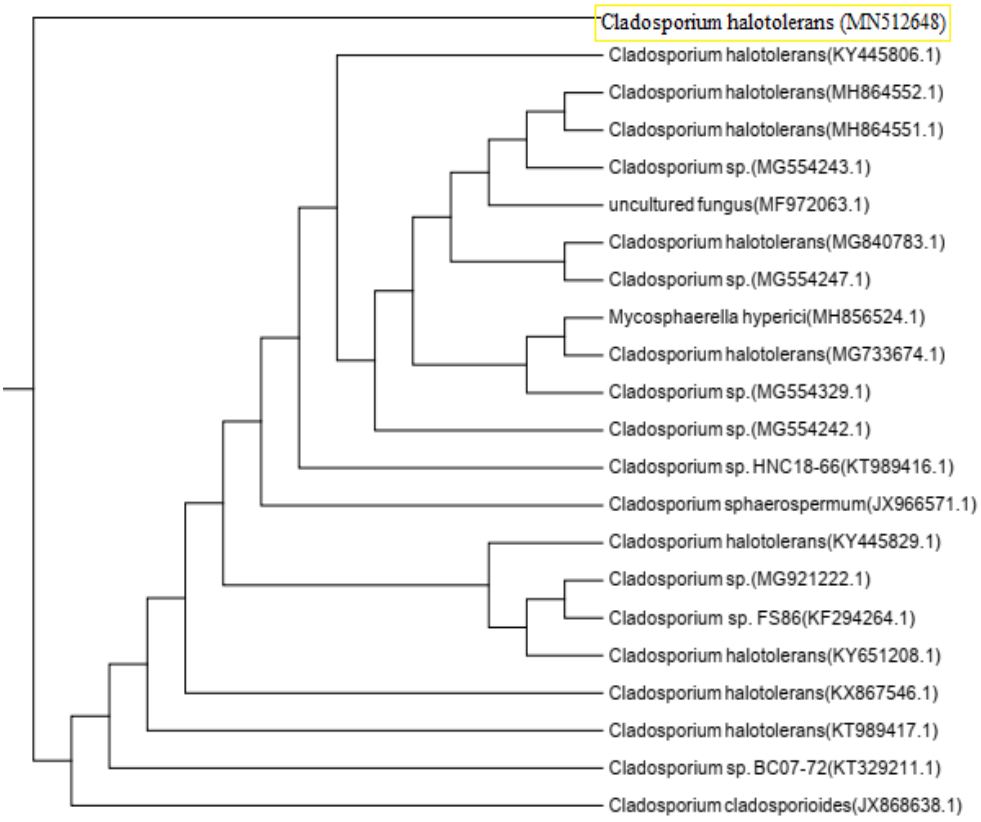

Fig. S3. Phylogenetic analysis tree of *Cladosporium halotolerans* (MN512648)

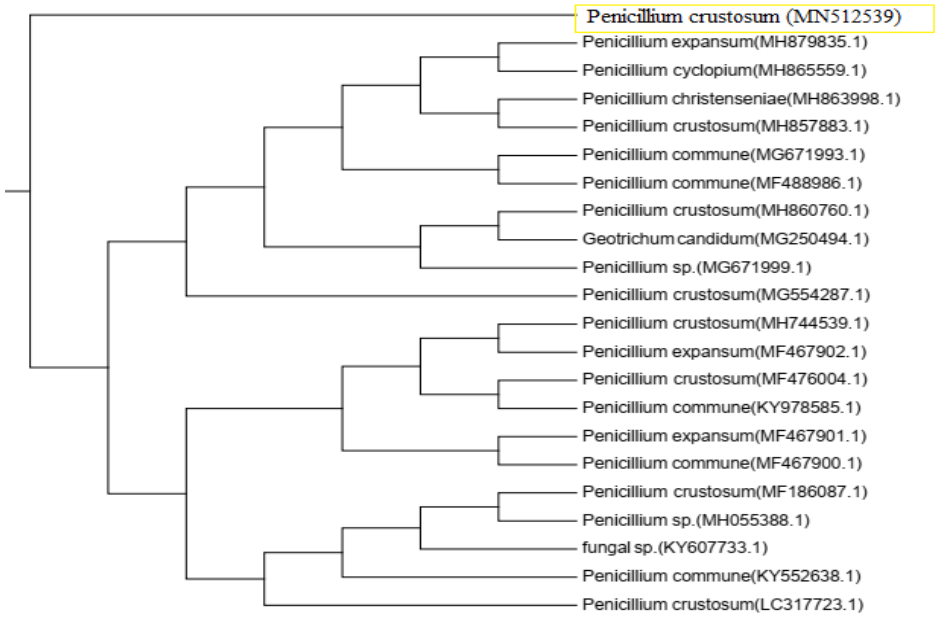

Fig. S4. Phylogenetic analysis tree of *Penicillium crustosum* (MN512539)

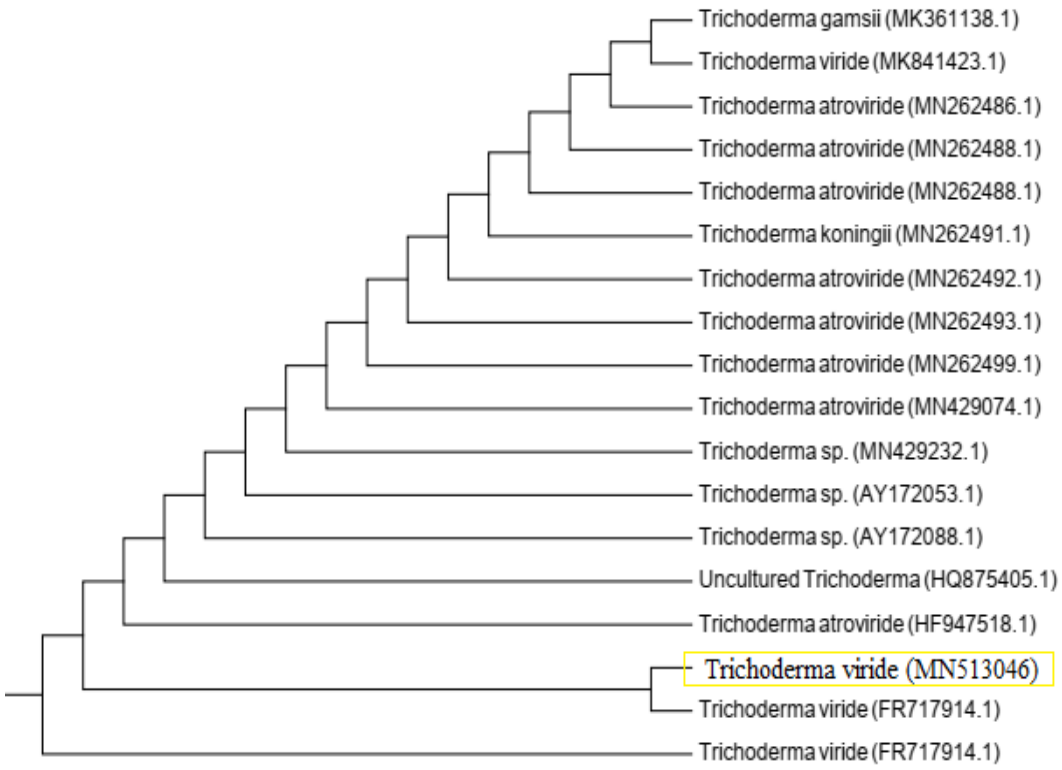

Fig. S5. Phylogenetic analysis tree of *Trichoderma viride* (MN513046)

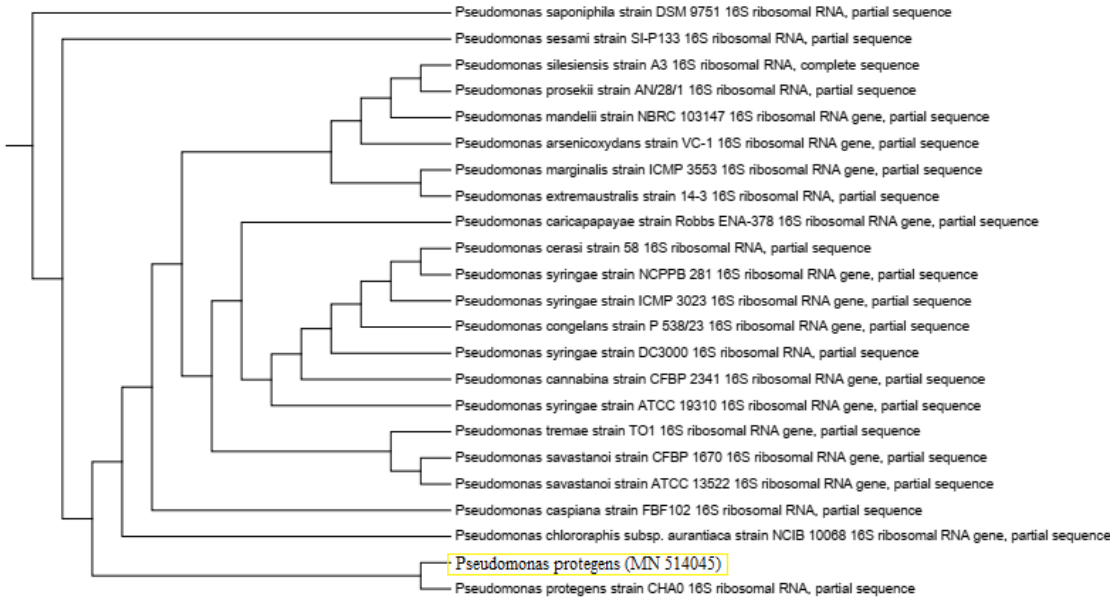

Fig. S6. Phylogenetic analysis tree of *Pseudomonas protegens* (MN514045)

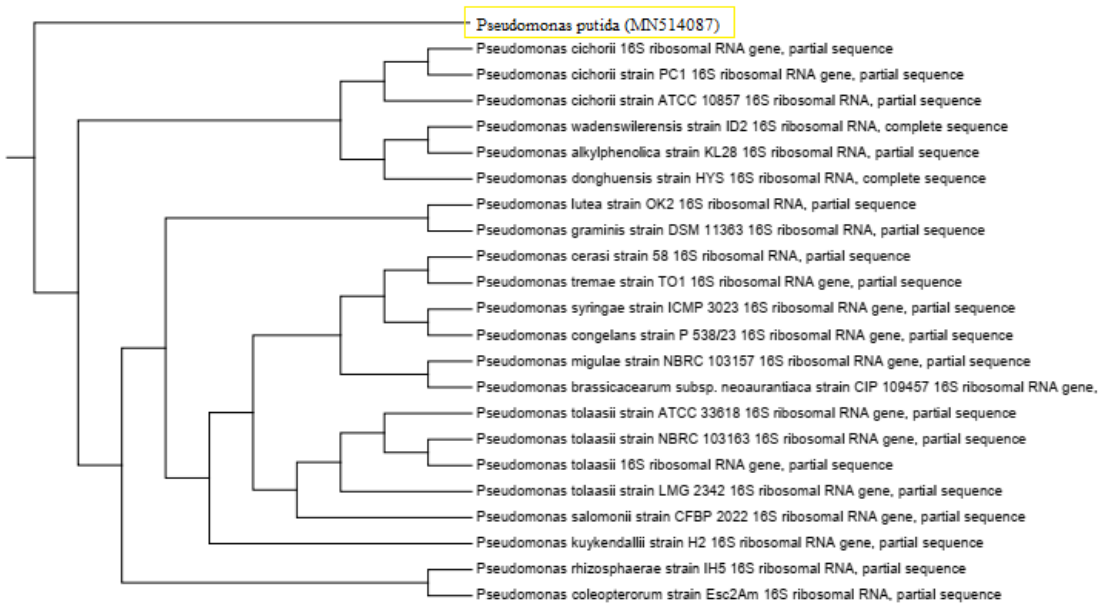

Fig. S7. Phylogenetic analysis tree of *Pseudomonas putida* (MN514087)

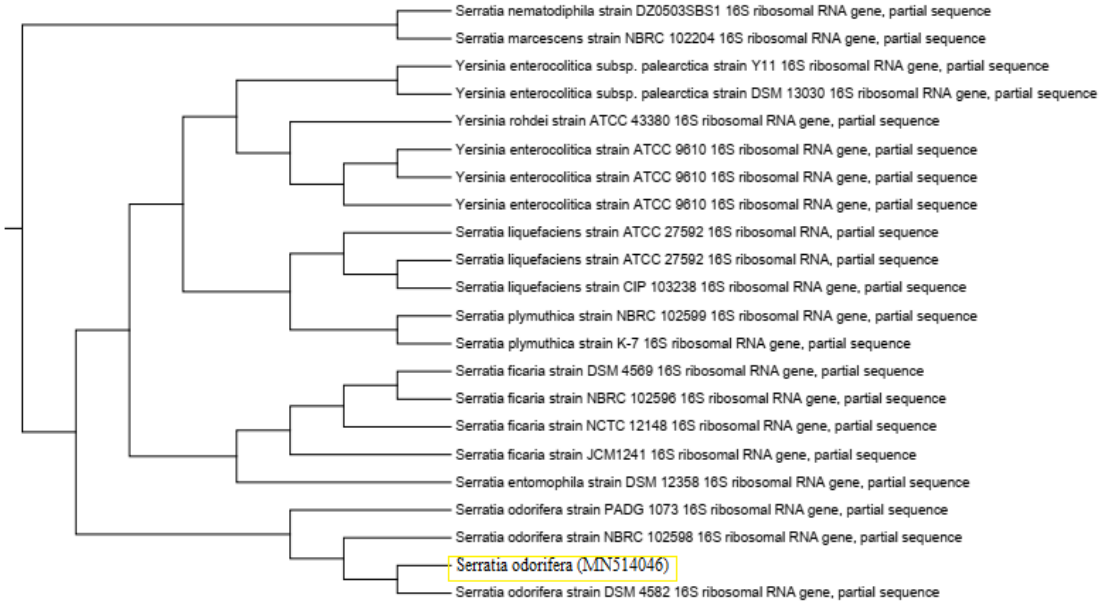

Fig. S8. Phylogenetic analysis tree of *Serratia odorifera* (MN514046)