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Antifungal, Antibacterial, and Interference Effects of Plant-Extracted Essential Oils Used for Mural Conservation at Buyeo Royal Tomb No. 1

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Abstract: Although subterranean tombs are largely protected from the external environment, the colonization of microorganisms threatens their conservation. Conventional biocides have negative effects on the environment, human health, and the sensitive materials in ancient tombs, especially painted murals. Therefore, we tested the biocidal effects of 11 plant-extracted essential oils (EOs) against two fungal strains and four bacterial strains isolated from Buyeo Royal Tomb No. 1, a World Heritage Site in South Korea. Oregano, clove bud, thyme, and cinnamon cassia EOs showed the highest antifungal and antibacterial activities. At concentrations suitable for practical application (3–10%), oregano and cinnamon cassia EOs exhibited the highest antifungal and antibacterial activities against the tested microbial strains. No variation in the surface properties and mineral composition was detected for the lithotype specimens (granite and gneiss) treated with the EOs at 1–10%. Low-concentration thyme and oregano EOs led to minimal color change in the painting layer specimens, whereas clove bud and cinnamon cassia EOs caused yellowing of the oyster shell white pigment at a concentration of 3–10%. Our results suggest that 3% oregano EO is a candidate biocide that could minimize the biological damage to and promote the conservation of ancient tomb murals.

Keywords: mural painting; microorganism; essential oil; interference test; painting layer

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1. Introduction

Subterranean cultural heritage sites, such as catacombs, ancient tombs, and other hypogean, sites are distributed underground worldwide and exhibit various forms and sizes [1,2]. Hypogean sites are characterized by a stable temperature, high relative humidity, and limited air circulation year-round [3–5], and they can thus be conserved over the long term in a state unaffected by the external environment. However, human factors, such as tomb robbery, excavation surveys, and tourist activities, have altered the unique environment of hypogeal sites [6,7] and have promoted the emergence and growth of microbes [8,9].

Hypogean sites are threatened by the growth of a variety of biological species, including cyanobacteria, green algae, diatoms, and various other types of microorganisms [3,10,11]. Notably, microorganisms cause discoloration and stains and form biofilms in ancient tombs, which are irreversible changes that induce aesthetic and structural damages, posing a major challenge for conservation [8,12–14]. The black or dark spots detected at the Takamatsuzuka and Kitora tumuli in Japan and the Etruscan hypogeal tombs in Italy were caused by *Acremonium* colonization [7,15]. The *Streptomyces* strain isolated from ancient Egyptian tombs and Buyeo Royal Tomb No. 1 in South Korea promoted discoloration and physical damage to murals [16–19].

Biocides based on alcohol, phenol, quaternary ammonium, and triazole compounds have mainly been applied in the past several decades to effectively remove microorganisms from cultural heritage sites [20,21]; however, these agents pose additional risks related to their toxicity, lack of degradability, and environmental persistence [22,23]. Certain biocides have been prohibited, as they were determined to be harmful to humans and the environment [24,25]. Moreover, biocides could serve as a source of nutrition for some microorganisms and actually promote their growth; the continued use of a biocide could result in the emergence of resistant strains and, by reducing competition, could provide an opportunity for colonization by a new microbial species [26–28]. To resolve these issues, it is essential to investigate environmentally friendly biocides [24,29,30]. Preconditions for an appropriate biocide application at a cultural heritage site include being harmless to the user, having minimal impact on the environment, and—most importantly—not interfering with the substrate [31–33].

Natural biocides have attracted substantial attention as an alternative to conventional chemical biocides in the conservation of cultural heritage sites [13,34,35]. Plant-based essential oils (EOs) are obtained through a variety of extraction methods from the flower, stem, leaf, and fruit [36]; they contain volatile aromatic compounds characterized by high volatility and lipid solubility [37,38]. Different EOs exert diverse effects, including antibacterial, antifungal, insecticidal, antioxidant, and anti-inflammatory effects [36,38]. Consequently, the application of plant-based EOs as green biocides in cultural heritage conservation has been investigated in recent years [22,39]. Several EOs have been shown to exert potent antibacterial and antifungal effects on microbes isolated from various materials, including stone, resin, and wood [24,29,34,40,41].

To date, the applicability of EOs in the conservation and restoration of hypogean sites has been tested using Greek aromatic herb, shrub, and tree extracts on microorganisms isolated from caves [42], and the effect of EOs has been tested on microorganisms isolated from the mural paintings in ancient tombs [43]. However, no EO has shown 100% efficacy across microbial species [4]. Therefore, research is required to develop essential oils that do not harm murals created with various techniques and in enclosed environments such as ancient tombs.

The aim of this study was to screen and evaluate the antifungal and antibacterial performance of various EOs against microorganisms isolated from Buyeo Royal Tomb No. 1—an ancient tomb mural from the Three Kingdoms period in South Korea. We further performed an interference test to evaluate the impact of the EOs on the mural materials.

2. Materials and Methods

2.1. Study Site

South Korea possesses ten ancient tomb murals, established in the 5th–15th centuries, most of which have been designated as Heritage Sites based on their historical and artistic value [44]. The Buyeo Royal Tombs—a tumulus of the Baekje era created between the late 6th century and the early 7th century—consist of seven ancient tombs. In 2015, the Buyeo Royal Tombs, one of the Baekje Historic Areas, were listed as a World Heritage Site by the United Nations Educational, Scientific, and Cultural Organization (UNESCO). Among the seven ancient tombs, Buyeo Royal Tomb No. 1 is the only tomb with mural paintings, which serves as evidence of the exchange between Baekje and Goguryeo.

The interior of Buyeo Royal Tomb No. 1 consists of aisles, a front room, and a main room; the mural paintings are located in the main room (Figure 1A,B). The main room is a stone chamber with a flagstone placed on the vertical wall. Large flagstones are set on each side—augen gneiss on the east wall, two-mica granite on the west wall, augen gneiss on the north wall, and granodiorite on the ceiling [45]. The murals were painted with pigments on the surfaces of these water-polished flagstones [46], a method known as “Jobyeokji”. Four guardian deities were painted on the walls using red, brown, black, white, and yellow pigments: a blue dragon on the east wall, a white tiger on the west wall, an orange phoenix on the south wall, and a black tortoise on the north wall. The ceiling has

paintings of lotus flowers and clouds (Figure 1C–E). The red pigment has been identified as cinnabar (HgS), the brown pigment as hematite (Fe_2O_3), and the white pigment as oyster shell white ($CaCO_3$) [46,47].

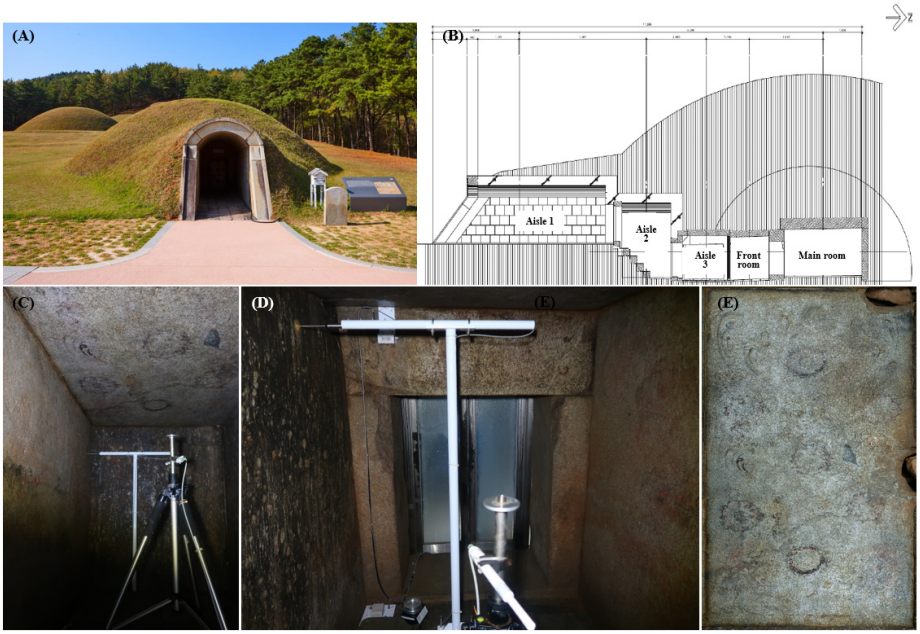

Figure 1. Photographs and structure of Buyeo Royal Tomb No. 1. (**A**) Panoramic view, (**B**) interior structure, (**C**) main room (orientation: south to north), (**D**) main room (orientation: north to south), and (**E**) a representative mural painting on the ceiling.

2.2. Microbial Growth Inhibition by Essential Oils

2.2.1. Selection of Standard Test Strains

Test strains isolated in microbial distribution studies of Buyeo Royal Tomb No. 1 in 2016, 2018, and 2019 [30,48,49] were selected based on the following criteria: (1) the strain should have been isolated in each study (2) in both domestic and international mural tombs (3) at a high microbial distribution rate (%), and (4) at least one species was selected per phylum.

The fungal strains were *Fusarium oxysporum* (XR_001936467.1; F1) and *Mortierella* sp. (KY773292.1; F2), and the bacterial strains were *Bacillus cereus* (NR_114582.1; B1), *Cupriavidus campinensis* (NR_025137.1; B2), *Streptomyces avidinii* (NR_041132.1; B3), and *Streptomyces cirratus* (NR_043356.1; B4). All strains were obtained from the Institute of Preventive Conservation for Cultural Property at the Korea National University of Cultural Heritage.

2.2.2. Selection of Essential Oils

With a focus on EOs extracted from plants, we performed a literature search of diverse natural biocides. Oregano is the most widely researched plant extract, followed by thyme, rosemary, eucalyptus, and tea tree. Based on the literature, a total of 11 EOs were selected for evaluation in the present study. Pure EOs without additional refinement, redistillation, or mixing were used in the experiments (Table 1).

2.2.3. Measurement of Antifungal and Antibacterial Activities of Essential Oils

We used the agar diffusion method to test the antifungal and antibacterial abilities of the EOs. Fungi were inoculated using the pour plate culture method. The microbial suspension was adjusted to 1.0×10^6 colony-forming units (CFU)/mL and mixed with potato dextrose agar (Difco, USA) for solidification on a petri dish (90 × 15 mm). The EOs were applied on a paper disc (8 mm diameter) and placed on the PDA; the disc was prepared to absorb 8 µL of oil. After culturing the specimen in an incubator for 5–7 days, we measured the diameter (mm) of the inhibition zone that formed around the paper

disc using vernier calipers (Figure 2A) and calculated the degree of inhibition using the Equation (1) [50].

Table 1. List of selected essential oils containing natural biocide substances.

Source of EO	Scientific Name	Extraction Method	Manufacturer
Eucalyptus	*Eucalyptus globulus*	Steam-distilled from leaves and small branches	Code 7545, Now Foods, Bloomingdale, IL, USA
Lavender	*Lavendula angustifolia*	Steam-distilled from flowering tops	Code 7560TL, Now Foods, Bloomingdale, IL, USA
Tea tree	*Melaleuca alternifolia*	Steam-distilled from leaves and twigs	Code 7625TL, Now Foods, Bloomingdale, IL, USA
Peppermint	*Mentha piperita*	Steam-distilled from aerial parts	Code 7585, Now Foods, Bloomingdale, IL, USA
Oregano	*Origanum vulgare*	Steam-distilled from dried flowering herb	Code 7494, Now Foods, Bloomingdale, IL, USA
Rosemary	*Rosmarinus officinalis*	Steam-distilled from tops of the plant	Code 7600, Now Foods, Bloomingdale, IL, USA
Clove bud	*Syzygium aromaticum*	Steam-distilled from the flower bud	What Soap, Korea
Thyme	*Thymus vulgaris*	Steam-distilled from the flower and leaves	What Soap, Korea
Grapefruit	*Citrus paradisi*	Cold-pressed from fresh fruit peel	Code 7553, Now Foods, Bloomingdale, IL, USA
Basil	*Ocimum basilicum*	Steam-distilled from the leaves	Code 7516, Now Foods, Bloomingdale, IL, USA
Cinnamon cassia	*Cinnamomum cassia*	Steam-distilled from the dried inner bark	Code 7530, Now Foods, Bloomingdale, IL, USA

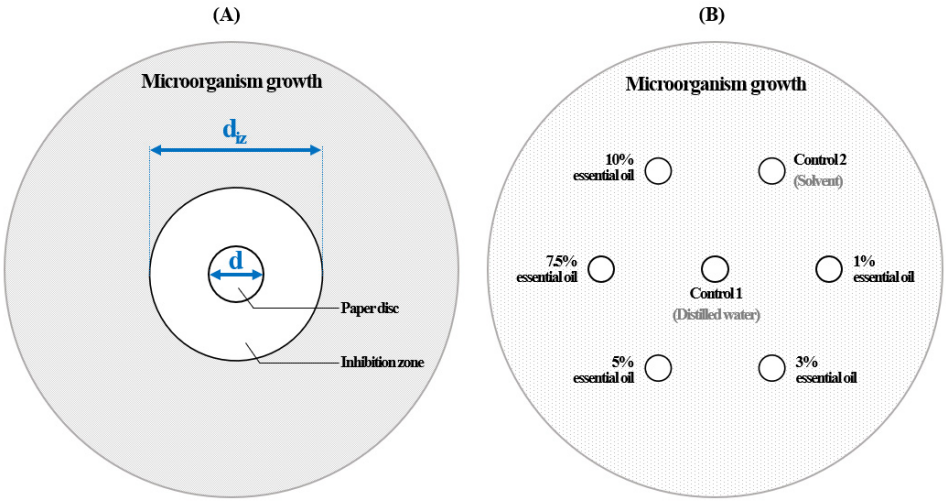

Figure 2. Evaluation of antifungal and antibacterial activities. (**A**) Measurements for the normalized width of the inhibition zone. (**B**) Location of the paper disc to test the antifungal and antibacterial activities of low-concentration essential oils.

Bacteria were inoculated using the spread plate culture method. First, after passaging on tryptic soy agar (Difco), a single bacterial colony was applied to tryptic soy broth (Difco), which was cultured in a shaking incubator (150 rpm, 30 °C) for 24–36 h. A suspension was produced from the activated bacterial strain at 1.5×10^8 CFU/mL, which was spread on tryptic soy agar in a petri dish (90 × 15 mm). The EOs were applied to a paper disc (8 mm diameter) and placed on the TSA; the disc was prepared to absorb 8 µL of oil. The specimen was cultured in an incubator for two days, the inhibition zone was measured (Figure 2A), and the degree of inhibition was calculated using Equation (1):

$$\mathrm{nw_{halo}} = \frac{(d_{iz} - d)/2}{2} \quad (1)$$

where d_{iz} is the diameter of the inhibition zone and d is the disc diameter. For both fungi and bacteria, data are expressed as the mean ± standard deviation of three measurements.

2.2.4. Measurement of Antifungal and Antibacterial Activities of Low-Concentration Essential Oils

Natural EOs are generally considered safe; however, certain components may cause skin irritation and hypersensitivity, as well as environmental hazards. Thus, caution must be exercised in their use [51,52]. Hence, the four EOs with the highest inhibitory effects (as determined in Section 2.2.3.) were selected and diluted to low concentrations using distilled water with 1% Tween 20 (CAS No: 9005-64-5, SAMCHUN, Korea), and 1%, 3%, 5%, 7.5%, and 10% (*v*/*v*) solutions of EOs were prepared.

The agar diffusion method was used to test the antifungal and antibacterial abilities of the EOs as described in Section 2.2.3; however, we used a 150 × 20 mm petri dish instead, and paper discs injected with EOs at different concentrations were placed at a set distance from each other (Figure 2B). The measurement of the inhibitory degree was then calculated as described in Section 2.2.3.

2.2.5. Statistical Analysis

One-way analysis of variance (ANOVA) was conducted to compare the differences in antifungal and antibacterial activities between EOs and low-concentration EOs. Duncan's multiple range test was conducted for post hoc analysis of the significant differences between the two groups at $p < 0.05$. All statistical analyses were performed using IBM SPSS Statistics ver.28.

2.3. Interference Tests on Low-Concentration Essential Oils

2.3.1. Stability of Stone Materials under Treatment with Essential Oils

Based on previous studies, we evaluated the interference of low-concentration EOs with stone materials, which can manifest as discoloration, EO residue, and crystal formation [34,53]. Granite and gneiss were selected for this experiment based on an analysis of stone types in the main room of Buyeo Royal Tomb No. 1 [45]. Stone specimens (2.5–5.5 g sourced from the Buyeo area) were washed with distilled water and dried in a 60 °C hot-air drier for 24 h. We analyzed a total number of 66 specimens for granite and gneiss. The EOs prepared in varying concentrations were placed in a 50 mL conical tube containing three specimens of the same stone, and the tube was shaken at ambient temperature (20 ± 2 °C) for 180 h. The controls were a mixture of distilled water with 1% Tween 20.

The changes in the specimen before and after the treatment with low-concentration EOs were assessed based on (1) surface observation, (2) weight measurements, and (3) mineral composition. The surface of the stone specimen was observed under a stereomicroscope (SMZ18, Nikon, Japan) at 7.5× magnification. After treatment, the specimens were washed 2–3 times with distilled water and dried in a 60 °C hot-air drier for 24 h before microscopic observation. We measured the weight of the specimens with an accuracy of up to 0.0001 g using an electronic scale (EX423, OHAUS, USA). We calculated the fluctuations in the weight of each specimen as follows: rate of weight change (%) = (difference in weight

before and after treatment)/(weight of specimen before treatment) × 100. The values were expressed as the mean of the three simultaneously treated specimens. We measured the changes in mineral composition following treatment of the stone specimens. Specifically, the 10% EO solutions were examined using an X-ray diffractometer (MiniFlex 600, Rigaku, Japan) that used a Cu-Kα ray (λ = 1.5418 Å) with a 40 kV acceleration voltage, 15 mA current, 2° per min injection speed, and 5–80° analytic range.

2.3.2. Stability of Painting Layers under Treatment with Essential Oils

A specimen of the painting layer was prepared by mixing the pigments cinnabar (HgS), hematite (Fe_2O_3), and oyster shell white ($CaCO_3$) [47,48] with 3% hide glue (Nakagawa Gofun Enogu, Japan), and the mixture was uniformly spread on a slide glass (76 × 26 × 1 mm) using a spatula. Low-concentration EOs were sprayed onto the vertically positioned specimen at a ~15 cm distance. The treatment followed the order of spray, dry, two sprays, and dry.

The estimated highest temperature and relative humidity in the main room of Buyeo Royal Tomb No. 1 were reported to be approximately 19–21 °C and 100%, respectively [31,54]. With the prediction of a future increase in temperature, the conditions of environmental degradation were set to 25 °C and 100% relative humidity. The specimen treated with the low-concentration EO was placed in a preconditioned wide-neck glass bottle, which was placed in a constant-temperature, constant-humidity chamber (WIG-155, DAIHAN Scientific, Wonju, Republic of Korea) for 4 weeks.

We subsequently examined the changes in each specimen based on (1) surface observations and (2) color measurements. The same point on the specimen was observed before and after the degradation treatment for comparison. The surface of each specimen was observed in detail under the stereomicroscope at a magnification of 30×. A spectrophotometer (ColorMate, Scinco, Republic of Korea) was used to measure the color of each specimen under D65 standard illumination and a 2° viewing angle. The mean of triplicate measurements was obtained, and the measured chromaticity values (L^*: lightness with 100; 0 = absolute black, 100 = absolute white, a^*: position between red/magenta [$a^* > 0$] and green [$a^* < 0$], b^*: position between yellow [$b^* > 0$] and blue [$b^* < 0$]) before and after the degradation treatment are expressed as L^*_1, a^*_1, b^*_1 and L^*_2, a^*_2, b^*_2, respectively; differences were calculated using Equations (2)–(4). The color difference (ΔE) was estimated using Equation (5) with a threshold of $\Delta E < 4$, which is the value generally applied by restorers [55].

$$\Delta L^* = L^*_1 - L^*_2 \quad (2)$$

$$\Delta a^* = a^*_1 - a^*_2 \quad (3)$$

$$\Delta b^* = b^*_1 - b^*_2 \quad (4)$$

$$\Delta E^*_{ab} = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (5)$$

3. Results

3.1. Microbial Growth Inhibition by Essential Oils

3.1.1. Antifungal Activity

Among the 11 EOs tested, oregano, clove bud, thyme, and cinnamon cassia showed equally potent antifungal activity against strains F1 (*F. oxysporum*) and F2 (*Mortierella* sp.). The oregano EO formed the largest inhibition zone (4.8–19.3 mm), followed by the cinnamon cassia EO (4.6–19.3 mm), thyme EO (4.3–19.3 mm), and clove bud EO (3.0–19.3 mm). The other EOs tested showed varying levels of antifungal activities depending on the fungal strain. With F1, lavender, tea tree, peppermint, and basil EOs formed small inhibition zones (0.3–0.4 mm), whereas no inhibition zone was observed for eucalyptus, rosemary, and grapefruit EOs. With F2, peppermint and rosemary EOs formed inhibition zones of 1.0–3.8 mm, whereas eucalyptus, lavender, tea tree, grapefruit, and basil EOs did not form an inhibition zone (Table 2).

3.1.2. Antibacterial Activity

Similar to the results for antifungal activity, oregano, clove bud, thyme, and cinnamon cassia EOs showed the most outstanding antibacterial activity against the four bacterial strains. Oregano and thyme EOs formed the largest inhibition zones at 6.7–19.3 mm, and clove bud and cinnamon cassia EOs formed inhibition zones of 2.6–9.4 mm and 8.1–15.6 mm, respectively. The antibacterial activities of lavender, tea tree, and peppermint EOs were comparable, and eucalyptus, rosemary, grapefruit, and basil EOs showed the lowest antibacterial activities. The mean diameter of inhibition zones was 7.9 mm for both B2 and B3, 7.2 mm for B4, and 3.9 mm for B1 (Table 2).

Table 2. Effect of tested essential oils (pure) on microbial strains isolated from Buyeo Royal Tomb No. 1.

Source of EO	Inhibition Zone (mm)					
	Strain F1	**Strain F2**	**Strain B1**	**Strain B2**	**Strain B3**	**Strain B4**
Eucalyptus	- *	- *	- *	1.0 ± 0.0	0.8 ± 0.0	1.0 ± 0.0
Lavender	0.3 ± 0.0	- *	0.8 ± 0.1	2.1 ± 0.2	4.3 ± 0.1	3.1 ± 0.4
Tea tree	0.3 ± 0.0	- *	0.8 ± 0.2	5.4 ± 0.1	5.3 ± 0.5	3.2 ± 0.2
Peppermint	0.4 ± 0.1	3.8 ± 1.2	0.9 ± 0.1	3.8 ± 0.0	4.0 ± 0.2	3.4 ± 0.3
Oregano	4.8 ± 0.2	19.3 ± 0.0	6.7 ± 0.9	19.3 ± 0.0	18.6 ± 0.5	18.4 ± 1.2
Rosemary	- *	1.0 ± 0.8	- *	1.1 ± 0.1	1.3 ± 0.2	0.9 ± 0.2
Clove bud	3.0 ± 0.0	19.3 ± 0.0	2.6 ± 0.0	9.0 ± 0.6	9.4 ± 1.5	6.8 ± 0.9
Thyme	4.3 ± 0.0	19.3 ± 0.0	7.5 ± 0.4	19.3 ± 0.0	19.3 ± 0.0	19.3 ± 0.0
Grapefruit	- *	- *	- *	0.3 ± 0.0	0.5 ± 0.0	0.6 ± 0.1
Basil	0.3 ± 0.0	- *	- *	1.9 ± 0.0	1.0 ± 0.1	2.6 ± 0.1
Cinnamon cassia	4.6 ± 0.1	19.3 ± 0.0	8.1 ± 0.3	16.8 ± 0.6	15.6 ± 0.8	13.6 ± 2.0
Control (solvent)	- *	- *	- *	- *	- *	- *

* -: <8 mm (strain is considered to be resistant to the essential oil). Data represent statistical differences in the antifungal and antibacterial activities of essential oils (pure) when $p \leq 0.001$.

3.2. Microbial Growth Inhibition by Low-Concentration Essential Oils

3.2.1. Antifungal Activity

The oregano EO formed an inhibition zone starting at a concentration of 5%, with larger inhibition zones found for F1 than for F2. The clove bud EO did not form an inhibition zone at any concentration. The thyme EO formed faint inhibition zones (0.2–0.3 mm), only at a concentration of 10%. The cinnamon cassia EO formed faint inhibition zones at a concentration of 3% and distinct inhibition zones ≥ 5.8 mm at a concentration of 5%; the inhibition zones were notably larger against F2 than F1 (Table 3, Figure 3).

Table 3. Effect of tested essential oils (low concentration) on microbial strains isolated from Buyeo Royal Tomb No. 1.

Source of EO	Strains	Inhibition Zone (mm)						
		1%	**3%**	**5%**	**7.5%**	**10%**	**Controls †**	
							Distilled Water	**Solvent**
Oregano	F1	- *	- *	- *	1.2 ± 0.5	2.0 ± 0.7	- *	- *
	F2	- *	- *	5.8 ± 0.0	5.8 ± 0.0	5.8 ± 0.0	- *	- *
	B1	- *	- *	0.2 ± 0.2	3.2 ± 0.6	3.3 ± 1.5	- *	- *
	B2	- *	1.3 ± 0.1	3.3 ± 1.2	7.5 ± 0.4	9.2 ± 1.6	- *	- *
	B3	- *	- *	2.1 ± 0.5	5.1 ± 1.7	5.9 ± 2.3	- *	- *
	B4	- *	0.3 ± 0.3	4.8 ± 2.3	7.0 ± 1.4	7.8 ± 1.9	- *	- *
Clove bud	F1	- *	- *	- *	- *	- *	- *	- *
	F2	- *	- *	- *	- *	- *	- *	- *
	B1	- *	- *	- *	- *	0.3 ± 0.0	- *	- *
	B2	- *	- *	0.4 ± 0.1	1.3 ± 0.5	3.3 ± 0.2	- *	- *

Table 3. *Cont.*

Source of EO	Strains	Inhibition Zone (mm)						
		1%	3%	5%	7.5%	10%	Controls †	
							Distilled Water	Solvent
Thyme	B3	- *	- *	0.1 ± 0.1	0.5 ± 0.2	0.8 ± 0.5	- *	- *
	B4	- *	- *	- *	0.5 ± 0.2	0.9 ± 0.1	- *	- *
	F1	- *	- *	- *	- *	0.3 ± 0.0		
	F2	- *	- *	- *	- *	0.2 ± 0.1		
	B1	- *	- *	- *	- *	0.3 ± 0.2	- *	- *
	B2	- *	- *	0.8 ± 0.0	1.3 ± 0.2	1.5 ± 0.2	- *	- *
	B3	- *	- *	- *	0.0 ± 0.1	0.3 ± 0.1	- *	- *
	B4	- *	- *	- *	0.4 ± 0.1	0.8 ± 0.2	- *	- *
Cinnamon cassia	F1	- *	0.3 ± 0.0	0.5 ± 0.0	0.8 ± 0.2	1.0 ± 0.0	- *	- *
	F2	- *	- *	5.8 ± 0.0	5.8 ± 0.0	5.8 ± 0.0	- *	- *
	B1	- *	1.2 ± 0.1	2.6 ± 0.3	3.7 ± 0.5	3.9 ± 0.1	- *	- *
	B2	- *	2.2 ± 0.1	3.3 ± 0.1	4.3 ± 0.4	4.6 ± 0.1	- *	- *
	B3	- *	3.2 ± 0.2	4.9 ± 0.5	6.5 ± 0.5	7.6 ± 1.0	- *	- *
	B4	- *	2.1 ± 0.5	4.1 ± 0.8	5.7 ± 0.6	7.1 ± 1.4	- *	- *

* -: <8 mm (indicates resistance to the essential oil). † Distilled water with 1% Tween 20. Data represent statistical differences in the antifungal and antibacterial activities of essential oils (low con-centration) according to oil concentration $p \leq 0.05$.

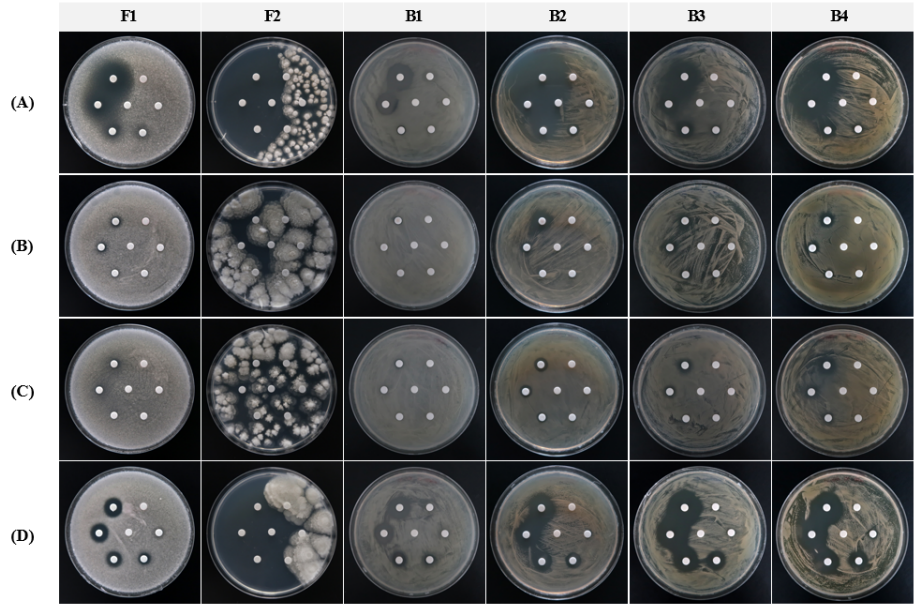

Figure 3. Evaluation of the antifungal and antibacterial activities of four essential oils at low concentrations: (**A**) oregano EO, (**B**) clove bud EO, (**C**) thyme EO, (**D**) cinnamon cassia EO. The arrangement of the paper discs (and their respective EO concentrations) is presented in Figure 2B.

3.2.2. Antibacterial Activity

The oregano EO at a concentration of 3% showed faint inhibition zones, which increased with increasing concentration (Figure 3, Table 3). The oregano EO showed the highest antibacterial activity against B2 and B4. The clove bud EO at a concentration of 5% formed faint inhibition zones, which increased slightly at concentrations of 7.5% and 10% (Figure 3, Table 3). Among the four EOs, thyme had the most inconsistent inhibitory effect, with inhibition zones formed at a concentration of 10% with B1, 5% with B2 and B3, and 7.5% with B4 (Figure 3, Table 3). The cinnamon cassia EO formed inhibition zones against all four bacterial strains at a concentration of 3%, and the diameter increased with

increasing concentration; the inhibition zones were notably large against B3 (3.2–7.6 mm) and B4 (2.1–7.1 mm) (Table 3, Figure 3).

3.3. Interference of Low-Concentration Essential Oils with Stone Materials

Based on our spectrophotometric observations, the granite specimens did not show any color changes after treatment with the low-concentration (1–10%) oregano EO, clove bud EO, thyme EO, cinnamon cassia EO, and the control groups. The rate of weight change for the granite specimens before and after treatment with low-concentration EOs was the lowest for oregano, followed by cinnamon cassia, thyme, and clove bud, and it was similar to or slightly higher than that caused by the controls (Table 4). In the specimens treated with EO concentrations of 10% (showing the highest effects across all concentrations), we detected quartz, mica, plagioclase, and albite both before and after the treatment (Figure 4). Newly formed crystals were not identified.

Table 4. Rates of weight change for the granite and gneiss specimens before and after treatment with low-concentration essential oils.

Source of EO	Concentration	Rate of Weight Change (%)	
		Granite	Gneiss
Oregano	1%	0.04 ± 0.02	0.06 ± 0.03
	3%	0.05 ± 0.02	0.32 ± 0.19
	5%	0.06 ± 0.04	0.03 ± 0.01
	7.5%	0.26 ± 0.13	0.04 ± 0.01
	10%	0.05 ± 0.02	0.04 ± 0.00
Clove bud	1%	0.97 ± 0.07	0.05 ± 0.02
	3%	0.69 ± 0.33	0.49 ± 0.25
	5%	0.22 ± 0.23	0.83 ± 0.56
	7.5%	0.46 ± 0.18	0.66 ± 0.29
	10%	0.77 ± 0.60	0.32 ± 0.09
Thyme	1%	0.09 ± 0.07	0.09 ± 0.10
	3%	1.19 ± 0.34	0.03 ± 0.01
	5%	0.07 ± 0.02	0.04 ± 0.00
	7.5%	0.43 ± 0.09	0.06 ± 0.04
	10%	0.15 ± 0.08	0.10 ± 0.05
Cinnamon cassia	1%	0.07 ± 0.02	0.04 ± 0.02
	3%	0.08 ± 0.01	0.08 ± 0.04
	5%	0.07 ± 0.02	0.06 ± 0.04
	7.5%	0.11 ± 0.02	0.34 ± 0.18
	10%	0.19 ± 0.19	0.41 ± 0.18
Control 2 (Solvent)		0.04 ± 0.01	0.03 ± 0.00
Control 1 (Distilled water)		0.09 ± 0.04	0.11 ± 0.02

Similarly, no color change was observed in the gneiss specimens after treatment with the four EOs at low concentrations (1–10%). The mineral composition before and after the treatment included quartz and feldspar, along with a small amount of mica (Figure 5). The rate of weight change caused by the EOs was the lowest for thyme, followed by oregano, cinnamon cassia, and clove bud (Table 4). The mean rates of weight change were 0.07%, 0.10%, and 0.19% for thyme EO, oregano EO, and cinnamon cassia EO, respectively, which was similar to that of the control groups (0.03–0.11%). The mean rate of weight change caused by the clove bud EO was 0.47%, which was slightly higher than that of the control groups (Table 4). It should be noted that the standard deviations for all weight change measurements indicate high variance in the results.

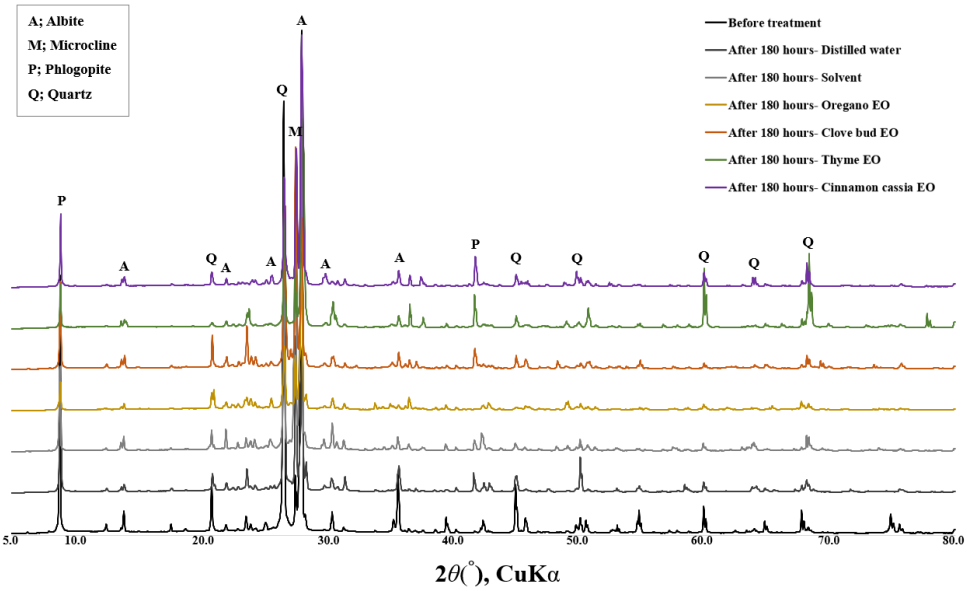

Figure 4. X-ray diffraction analysis of granite specimens before and after treatment with 10% low-concentration essential oils.

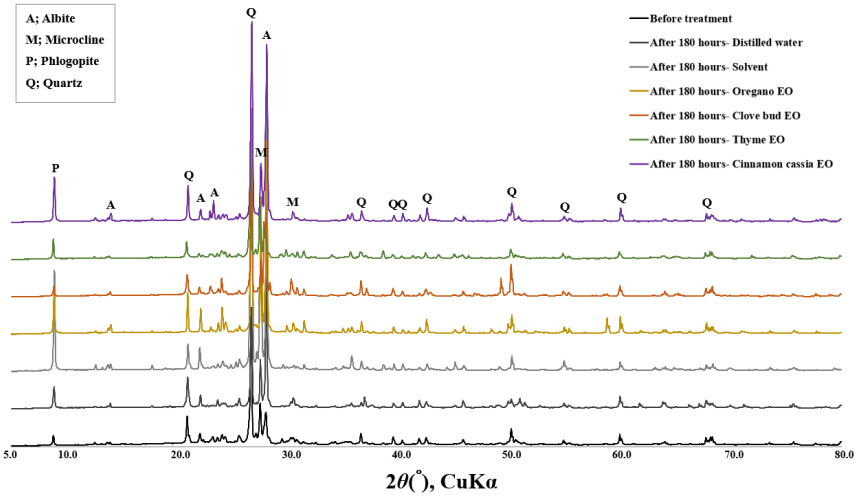

Figure 5. X-ray diffraction analysis of gneiss specimens before and after treatment with 10% low-concentration essential oils.

3.4. Interference Tests of Low-Concentration Essential Oils on Pigments

Pigment swelling and leaching were not observed in any painting layer specimens treated with EOs. Cinnabar specimens treated with oregano, clove bud, and thyme EOs showed a color range of 0.57 to 1.32 with $\Delta E < 2$ for all three EOs and the control group (untreated and solvent-treated). The L^*, a^*, and b^* values of specimens treated with oregano and thyme EOs increased in a concentration-dependent manner. The L^*, a^*, and b^* values of specimens treated with clove bud and cinnamon cassia EOs all tended to decrease; the color difference of specimens treated with cinnamon cassia EO ranged from 1.25 to 3.18, which was the highest for all EOs, with $\Delta E > 3$ at a concentration of 10% (Figure 6A).

The color difference of the hematite specimens was $\Delta E = 2.69$ for the untreated group and $\Delta E = 1.02$ for the solvent-treated group. The color difference was affected by the b^* value (increased by 2.41) in untreated specimens and the L^* value (decreased by –0.48) and b^* value (increased by 0.52) in solvent-treated specimens. The mean color difference of hematite specimens was the highest for the cinnamon cassia EO (ΔE 4.06), followed by the thyme EO (ΔE 3.37), clove bud EO (ΔE 2.99), and oregano EO (ΔE 2.89), with the color difference mostly affected by the b^*, L^*, and a^* values (in descending order of effect

size). Cinnamon cassia EO-treated specimens with $\Delta E < 3$ had a mean change of $L^* = -1.95$, $a^* = 1.64$, and $b^* = 3.10$, and thyme EO-treated specimens had a mean change of $L^* = -1.85$, $a^* = 1.44$, and $b^* = 2.33$ (Figure 6B).

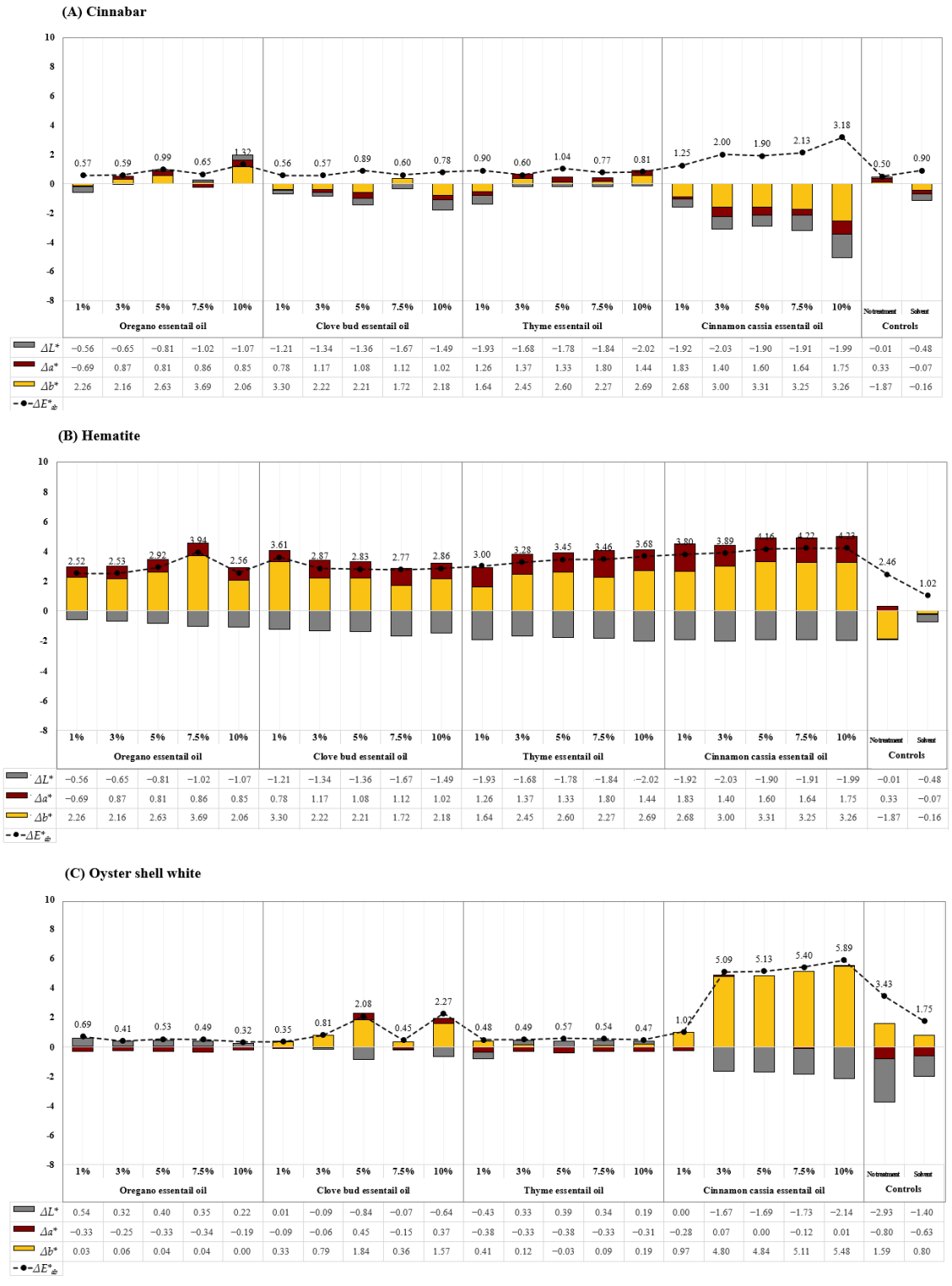

Figure 6. Color difference values (ΔL^*, Δa^*, Δb^*, and ΔE^*ab) before and after treatment of pigment specimens with low-concentration essential oils.

Oyster shell white specimens treated with oregano and thyme EOs showed a slight color change of $\Delta E < 1$. In contrast, specimens treated with cinnamon cassia EO showed a

maximum color difference of ΔE = 5.89. Specimens treated with 3–10% cinnamon cassia EO showed an overall yellowing effect, which was associated with an average increase of 5.06 in the b^* value (Figure 6C). Yellowing was also observed for specimens treated with clove bud EO, though to a lesser degree. Oyster shell white specimens of the control group developed mold and hence were considered unsuitable.

4. Discussion

Among the tested EOs, oregano, clove bud, thyme, and cinnamon cassia exhibited the most potent antifungal and antibacterial activities. However, for an EO to effectively conserve the murals of ancient tombs, we also considered how the EOs functioned in the closed environment of the ancient tomb and their impacts on mural materials (e.g., pigment, medium, substrate) [29,30,56]. Accordingly, we analyzed the inhibition of microbial growth caused by the four most potent EOs following dilution to low concentrations. Oregano and cinnamon cassia EOs showed the highest antifungal and antibacterial activities against the six microbial strains, with inhibitory effects observed from a concentration of 3%. In previous studies, the oregano EO extracted from the aerial parts of the plant was found to inhibit the growth of bacterial strains of *Bacillus, Achromobacter, Sinorhizobium, Paenibacillus, Rhodococcus,* and *Stenotrophomonas* and fungal strains of *Penicillium, Clonostachys, Fusarium, Doratomyces*, and *Acremonium* at a concentration as low as 0.1% [42]. The oregano EO was also reported to exhibit antimicrobial activities against fungal strains isolated from stone and wood materials (*Aspergillus niger, A. clavatus, Penicillium* sp., and *Fusarium* sp.) at a concentration of 7.5% [57] and against the wood-destroying fungal strains *Postia placenta* and *Trametes versicolor* at concentrations of 0.25–1% [58]. The antimicrobial activities of 7.5% oregano EO against *Fusarium* sp. observed in that study are consistent with our findings. In light of these findings, oregano EO extracted from the dried flowering herb may inhibit ascomycetes isolated from ancient tombs. Cinnamon cassia EO at concentrations of 0.1–0.5% was reported to inhibit the growth of *B. safensis* and *S. rochei* isolated from limestone monuments [59]. These studies reported inhibitory effects at lower concentrations of EOs than those reported in the current study, which may be related to differences in the method of EO extraction and the plant parts used, along with the type and content of emulsifier used. The use of emulsifiers in the dilution of EOs is unavoidable [60,61]. Although most researchers use dimethyl sulfoxide (DMSO) and ethyl alcohol [22,56,57], DMSO could have a deteriorating effect on archaeological materials and is hazardous to human health [61], and ethyl alcohol could serve as a nutrient source for microorganisms [26]. Thus, the selection of an appropriate emulsifier is a critical consideration in the application of EOs in ancient tomb murals. Although numerous previous studies have reported high antifungal and antibacterial effects of thyme and clove bud EOs [34,40,58], these EOs displayed low inhibitory effects in the present study. This discrepancy could stem from the use of undiluted EOs in previous studies or the evaluation of their inhibitory effects at concentrations of 10%.

We further evaluated the impact of low-concentration oregano, thyme, clove bud, and cinnamon cassia EOs on stone and painting layers representative of the murals of Buyeo Royal Tomb No. 1. None of the EOs affected the surface property or mineral composition of the granite and gneiss specimens. Although some studies have shown that biocide treatment causes minimal changes in the weight of stone compared with distilled water treatment [34,53], our findings suggest that clove bud EO may remain on some granite and gneiss specimens. On Lascaux cave paintings, *F. solani* fungal invasion was caused by residues of previously treated biocides that acted as nutrient sources for the microorganisms [28]. Similarly, biocide treatment may alter the diversity of the microbial community [62]. Hence, caution must be exercised for potential new challenges that may arise as EOs remain on ancient tombs.

The stability of low-concentration EOs on painting layers was primarily assessed based on the color change in the oyster shell white pigment, which can be readily detected by spectrophotometric or microscopic observation. The clove bud EO caused yellowing at

a concentration of 5%, although this fell below the chromaticity criterion. The cinnamon cassia EO caused yellowing at a concentration of 3%. These effects are presumed to be associated with the inherent yellow coloration of these two EOs. Moreover, 7% clove bud and 1% cinnamon cassia EOs are known to induce the discoloration of pigments as well as fabric and paper materials [63]; thus, their direct use on murals should be avoided. In contrast, 1–10% thyme and oregano EOs led to color changes below the threshold ($\Delta E \leq 4.0$) in the oyster shell white, cinnabar, and hematite pigments, thereby exhibiting the highest stability on painting layers.

Given the wide variety of factors related to the production of EOs, including the extraction method and emulsifier selection, it is difficult to determine the optimal method of application (e.g., brushing vs. spraying), optimal amount of EO, and potential change in stability of the various layers of the murals over time. Future studies should focus on developing and standardizing an experimental procedure for the application of EOs to ancient tomb murals, and a long-term study should be conducted on the stability of EOs on mural specimens while considering the characteristic conditions of ancient tombs.

5. Conclusions

In this study, we investigated the potential microbial growth inhibition by eucalyptus, lavender, tea tree, peppermint, oregano, rosemary, clove bud, thyme, grapefruit, basil, and cinnamon cassia EOs. Oregano, clove bud, thyme, and cinnamon cassia EOs showed the strongest inhibitory effects on microorganisms commonly isolated from ancient tomb murals. We analyzed the stability and microbial growth inhibition of these four EOs at low concentrations (1%, 3%, 5%, 7.5%, and 10%) and found that the oregano EO exhibited the highest antifungal and antibacterial activities, as well as the best stability with mural materials. The cinnamon cassia EO also exhibited high antifungal and antibacterial activities but caused pigment discoloration at a 3% concentration. The thyme EO showed adequate stability with mural materials but low levels of microbial growth inhibition, whereas the clove bud EO showed low levels of microbial growth inhibition and inadequate stability. Therefore, our results suggest that 3% oregano EO has great potential as a natural biocide against microorganisms colonizing the mural paintings of Buyeo Royal Tomb No. 1 and other similar ancient tombs. However, the production techniques of ancient tomb murals and the preservation environment of each mural vary among cultural heritages. Therefore, further long-term and comprehensive studies must investigate the methods of EO treatment, the stability of mural materials after treatment, and the field application evaluation in ancient tomb environments. This work will help to promote the application of EOs as an environmentally friendly technique to conserve ancient tomb murals from microbial damage.

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