Intervención

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Comparative Evaluation of the Antifungal Activity of Extracts of Nurite (*Satureja macrostema*), thyme (*Thymus vulgaris*) and the Commercial Brand Citrik® in the Conservation of Paper Documents

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Translated by Richard Addison

ABSTRACT

This research seeks to investigate the use of chemical substances used to control biodeterioration in industrial paper documents, proposing less harmful alternatives of vegetable origin. Two essential oils (EO) and a commercial disinfectant were evaluated against four predominant fungal strains in test tubes belonging to the collection of the Public Library of the State of Jalisco "Juan José Arreola" (BPEJ), Mexico. The application was made inside a fumigation chamber by means of nebulization. The results were evaluated according to the growth halo and number of colonies present in the Petri dishes; it was found that *Citrik*® Restoration and nurite (*Satureja macrostema*) are effective at concentrations higher than 5%, while thyme extract (*Thymus vulgar-*

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is) shows favorable results from 2%, having efficacy in the decrease of *Aspergillus* sp., *Cladosporium* sp., and *Penicillium* sp.

KEYWORDS

preservation, essential oils, antifungal, *Thymus vulgaris*, *Satureja macrostema*, fumigation

INTRODUCTION

In the field of archive, library, and museum conservation, aromatic plants like cedar *(Cedrela odorata)* and cypress *(Cupressus sempervirens)* were used to repel insects, while toxic plants, such as gentian *(Gentiana lutea),* were used to poison bookbinding glue (Borrego-Alonso, 2015, p. 263). However, these practices came to be replaced by chemical products, whose transmission depended more on the practicality and ease of obtaining/applying than on a rigorous analysis of the method of action and the behaviour of the substrate.

The current state of the field reveals the scarce research made so far on the limited evaluation and use of products of natural origin applied to items of cultural heritage. Only a few authors refer to their potential application in the discipline of cultural property conservation (Morales et al., 2013, pp. 152-154; Rio, 2019, pp. 28-29; Ali, 2020, pp. 81-85; Palla et al., 2020, pp. 2-8; Russo & Palla, 2023, p. 11). In Spain, Italy, and Cuba studies have been more frequent and deal with objects of diverse materiality, such as photography, wood, stone, mural painting, among others, addressing issues related to their biodeterioration, a fundamental aspect for the conservation of the material addressed here, which is paper.

On the other hand, the disinfectants of chemical-industrial origin most commonly used to counteract this type of biological agents usually create, in addition to resistance to microorganisms, remanence in the support, most of which are harmful to human health (user-operator) and the environment. They also cause physicochemical changes in the substrate to which they are applied. Nevertheless, the biodegradable substances on the market in general, and in the field of conservation in particular, offer highly variable concentrations on using very limited materials and fungal species. Therefore, the *Citrik*® Restauración disinfectant will be the frame of reference for this research, as it is commonly used in conservation processes and in the Public Library of the State of Jalisco "Juan José Arreola" (BPEJ), which provided for academic purposes

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-specifically for the development of this work- industrial paper supports categorized as discarded specimens.

Based on the previously identified needs outlined in the BPEJ, specific supports that exhibited distinctive characteristics that set them apart from the rest, were isolated for analysis. Consequently, the study focused on examining the fungal spectrum and evaluating the inhibitory effects of the applied substances. Particular attention was given to documenting the most significant fungal damage to the paper, enabling the identification of the fungal species present. The findings then guided efforts to address the most prevalent genera within the collection.

This research aims to explore plant-based antifungal alternatives for application in the preservation of documentary heritage, while also leveraging insights from multidisciplinary fields such as biotechnology. Specifically, the study seeks to transfer knowledge about the properties and applications of essential oils (EO) derived from thyme (*Thymus vulgaris*) and nurite (*Satureja macrostema*) into the conservation domain. By doing so, it aspires to open new avenues for research and innovation in heritage preservation.

PAPER BIODETERIORATION

The documentary heritage studied consists of materials such as paper, adhesives (both organic and inorganic), cloth, and cardboard, all of which contain high levels of cellulose, glucose, lignin, starch, and proteins. Over time, these materials degrade, leading to partial or total loss, a process that is often unavoidable, but can be slowed or mitigated. To prevent or slow down this deterioration, it is essential to identify the two primary factors that contribute to it: intrinsic and extrinsic.

The first is related to the raw material; that is, to the constitution of the support: pulps, inks, pigments, binders, etc. The second is associated with the external environment and context; with the climatic changes of temperature, lighting, high relative humidity and pollution; those that occur within the building, for example, the lack of maintenance in the building, the poor state of conservation of the collection, the accumulation of dust, the presence of animals or microorganisms; as well as human interaction, be it directly or indirectly.

The neglect of these conditions can, on some occasions, further the proliferation of microorganisms[1] that use paper as a food

[1] The most common species are: *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., *Rhizopus* sp. (Sarasti, 2018, p. 61).

source, and which can have negative consequences on human health, causing respiratory diseases, skin irritations and allergies, depending on the time of exposure and the user's health state. It has been reported that some isolated genera[2] of fungi in documents are capable of producing volatile organic compounds and mycotoxins, some of them associated with strong odours of humidity and old material (paper or cloth), which are highly irritating to the respiratory system (Hidalgo & Borrego, 2006, p. 3).

The appearance of the alterations caused by biodeterioration depends on the type of substrate that the objects are made of, so that a piece of compact stone will have wider critical limits compared to those of organic origin such as paper. Therefore, "[...] biodeterioration cannot be considered as an isolated phenomenon since other deterioration processes of chemical, physical, or physicochemical type occur in parallel, with which it is closely correlated [...][3]" (Borrego-Alonso, 2015, p. 260).

The presence of fungi in documents causes three types of alterations: chemical, mechanical, and chromatic (Rivero, 2014, p. 3). The microbiological agents and the main factors that favour the chemical degradation of paper are acid, alkaline, and enzymatic hydrolysis, which cause the degradation of cellulose fibers.

With regard to mechanical affections, these organisms weaken the support fibers when they absorb water, as they grow and rapidly branch out to form colonies between them. In this sense, the hyphae penetrate forming a network of tunnels in the agglutinating area, whereby the cells associated with each other adhere to the surface through a three-dimensional structure with channels and pores - used both for intercellular communication and for the maintenance of the entire community - ideal for developing and subsisting together (Nazzaro *et al.* 2019, p. 5; Ali, 2020, p. 80). In addition, during their development they produce metabolic water, which increases the humidity in the material and, possibly, the proliferation of other biological agents.

Regarding chromatic alterations, the fungi become pigmented as a result of metabolism, mycelial growth and the release of acid and enzymatic residues (Fernandez & Maqueira, 2013, pp. 24-25). As a result, stains of various colours are generated depending on their stage of development, nutrients, the type and pH of the substrate, and the presence of other organisms and environmental factors on the support, which makes their removal very difficult

[2] *Aspergillus* sp., *Cladosporium* sp., *Chaetomiun* sp., *Trichoderma* sp. (Hidalgo & Borrego, 2006 p. 3).
[3] Editorial translation.

and presents a challenge for conservation. As a result of the degradation of cellulose caused by this metabolism of fungi, the paper acquires a porous and lax appearance; this is combined with the acidification of the environment in which they develop.

Disinfection methods

Among the methods used to disinfect cultural objects, we see frequent use of toxic chemicals such as ethylene oxide, orthophenyphenol, pentachlorophenol, and phostoxin, some of which, although they have a broad biocidal spectrum, are still banned for certain uses in several countries in Latin America and Europe (Sarasti, 2018, p. 61). Besides causing respiratory tract and eye irritation, they can lead to dermal lesions, damage to the central nervous system, as well as posing an increased risk of cancer,;they are also difficult to degrade and, therefore, persist in the support and the environment.

The long-lasting reminiscence left on the materials can be reduced by careful washing; however, not all documents are capable of withstanding such a process after the application of the substances under discussion. This can degrade the cellulose and cause depolymerisation of the materials, loss of flexibility, colour changes, accelerated ageing, increased corrosion of iron gall inks, among others (Valentín & García, 1999, p. 88; Rivero, 2014, pp. 9-10). To make matters worse, in addition to the above, these are highly toxic which can lead to having to increase the concentration. Cappitelli, Cattò, and Villa put it in the following way:

> Despite their toxicity, traditional biocides are still widely used, however, they are short-lived and often need to be repeated frequently and can cause resistance in biological agents, modifying internal structures and favouring the growth of more harmful organisms (recolonization)[4] (Cappitelli, Cattò and Villa, 2020, p. 4).

Currently, there are disinfection methods that propose to be eco-sustainable in the area of conservation, restoration, and other fields. In the market, the commercial chemical disinfectant *Citrik*® Restauración, made from citrus extracts (grapefruit and orange), is efficient for the inhibition of bacteria, fungi, yeasts, and spores on various substrates and surfaces. According to the product's tech-

[4] Editorial translation.

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nical data sheet, the disinfection effect on the fungal strain *Aspergillus niger* is recognized but doesn't make any reference to other genera frequently found in documentary heritage (Marco Polo Alta Tecnología en Preservación, 2022, p. 21).

Likewise, conservation-restoration disciplines are incorporating methodologies that apply combined treatments, such as anoxia, where the amount of oxygen is reduced to minimum concentrations and/or replaced with other types of gases, such as nitrogen or ethylene oxide (Paz & López, n. d., p. 2). Although anoxia seems to be a viable option for the application of chemicals, most of the time it ends up being a high-cost process due to the equipment and infrastructure required, which is highly dependent on the size of the piece. It should be noted that such treatment becomes ineffective for certain microorganisms, as the anaerobic condition of several of them allows them to survive this method, leaving spores in a transition period to a new condition. One which is a dormant state in which the cells have been damaged but are not completely dead and can remain so until the conditions are right for them to develop again.

It should be noted that any application should be done after performing first a deep cleaning of the document, since this removes any dust and hyphal remains from the surface. However, the growth of microorganisms occurs between the fibers of the paper, not only on the surface, so these two actions, cleaning and inhibition, must be complemented along with careful control of environmental conditions.

Characteristics of the EO used for the study

The activity of an essential oil (EO) (*Satureja macrostema*, *Thymus vulgaris*, *Citrik*® Restauración) varies depending on its exact chemical composition. This change depends on factors such as environmental conditions, type of species and phenological stage of the plant, the part used to extract the essence, time of harvest, extraction method, geographical area, among others.

Satureja macrostema is a shrubby plant with a minty odor and orange-red flowers (Figure 1). It grows in the Mexican states of San Luis Potosí, Jalisco, Michoacán, and Oaxaca. It is of ritual, ornamental, and medicinal use in San Miguel Mixtepec, a Zapotec community in the Central Valleys of Oaxaca (Ortega-Ortega & Vázquez-García, 2014, p. 71). It has several common names, such as nurite, tabaquillo, hierba de borracho, truche, garañona, té de monte, atóchietl, poleo, *cuencuenzpatli*, among others (Alonso,

2009, pp. 17-18). Several species of the genus *Satureja* have been reported to possess antifungal components (Damián-Badillo, Salgado-Garciglia, Martínez-Muñoz, & Martínez-Pacheco, 2008, p. 32). This is the case of *Satureja macrostema*, recognized for its biocidal effects against organisms of the genus *Colletotriuchum, Fusarium, Verticilum*, and *Phytophthora*; it also inhibits the growth of bacteria such as *Salmonella* sp*., Escherichia coli* sp*., Shigella* sp*., Staphylococcus aureus*, and *Streptococcus* sp. (Salgado, 2004, in Carrillo, 2009, p. 16).



FIGURE 1. *Satureja macrosterma* plant in reproductive stage (Source: Ortega-Ortega & Vázquez-García, 2014, p. 73).

Similarly, another known species with antimicrobial activity due to its AE is the *Lamiaceae*, or *Labiateae*, represented worldwide by about 3 000 species. This family includes herbaceous, annual, and perennial plants that are traditionally used as aromatics (Russo & Palla, 2023, p. 4). Particularly, the EO of *Thymus vulgaris*, or thyme, is one of the proven and most studied extracts, both in antiquity and in modern fields such as the field of biotechnology, as an inhibitor in the development of microorganisms, to mention a few: *Alternaria alternata*, *Fusarium tricinctum*, *Aspergillus* sp. species*, Phomopsis helianthi*, and *Cladosporium cladosporioides* (Soković *et al.*, 2009, p. 14; Palla *et al.*, 2020, pp. 2-7). This indicates that it not only shares chemical, aromatic and medicinal properties with other extracts, but also stands out for its characteristic of being a clarified oil; i.e., free of impurities and colorless, in the same way as *Satureja macrostema*.

Plants have antibacterial and antifungal properties that they naturally produce in cell tissues as a defence mechanism against attacks by microorganisms. Therefore, EO have been studied throughout history in different fields, starting with traditional medicine and, nowadays, their analysis has been further developed in biotechnology (Nazzaro *et al.,* 2019, p. 7). It is important to highlight their effectiveness against a wide range of microorganisms, even at low concentrations, in a wide pH range, but also their solubility in water and alcohols, low cost and low toxicity to humans and the environment (Borrego-Alonso, 2015, p. 262).

In general terms, the concept of an *antifungal agent* encompasses any substance capable of causing damage to the cellular structures of a microorganism, preventing its development and disrupting its ability to survive, grow, and/or reproduce (Gamboa, 2015, p. 6). According to Armas, Márquez, and Pretell "Plant tissues have defence mechanisms when exposed to certain stresses; one of the responses is the release of volatile compounds that are toxic against certain pathogens[5]" (2011, p. 124). Among the secondary metabolites with antifungal activity are those from the liquid-volatile fraction, which contain the substances responsible for the aroma of plants or EO. Some of these components are terpenes, phenols, aromatic aldehydes, ketones, esters, acids, and alcohols (Gamboa, 2015, p. 6). Generally, the majority elements determine the biological properties and are known as *chemotypes* (Agencia Española de Medicamentos y Productos Sanitarios [AEMPS], 2016).

The predominant chemotypes of both *Thymus vulgaris* and *Satureja macrostema* essential oils are thymol and carvacrol, which are terpenoids attributed with the strongest antifungal and antimicrobial action (Galovičová *et al.,* 2021, p. 1). Due to their phenolic structure, they are capable of destroying cells by altering the permeability of the cell membrane and any resulting leakage from the constituent parts (Samah & Hoda 2021, p. 1030).

As for citrus fruits, most of them are characterized by their high antioxidant content and low toxicity. Both *Citrus sinensis* (orange) and *Citrus paradisi* (grapefruit) possess antifungal, antimicrobial, and antiallergenic properties. In general terms, linalool is a volatile monoterpene compound common in the EO mentioned, whose natural form is common in many flowers and aromatic plants that all share almost the same properties.

[5] Editorial translation.

From a general perspective, it is possible to observe that substances with similar molecules tend to dissolve easily into each other. In this context, both EO and alcohol are hydrocarbon chains; however, alcohol exhibits a duality in its molecules, presenting both polar and non-polar regions in its structure. This particularity gives it the ability to interact with a wide range of compounds. In contrast, oil is also composed mostly of hydrocarbon chains but is characterized solely by non-polar molecules. Therefore, essential oils have almost similar functional groups that attract each other and favour the formation of solutions where both substances are homogeneously integrated.

According to the proposed method for the application of disinfectant substances, the constituent material of the test tubes, such as the inks, did not present a risk of solubility, since the paper was not impregnated. However, solubility tests were carried out in order to confirm the dissolution of EO in isopropanol at different concentrations until observing whether both substances were miscible, separated or presented some state of emulsification, color change, etc. It is worth mentioning that isopropanol alone is not considered a sporicide (Rodríguez, 2006).

Part of the defence mechanism (Figure 2) of natural products from plants operates when the tissue experiences some stress, where some cells become toxic to others to defend against any disturbance. However, the general function of EO aims to inhibit the growth of microorganisms by denaturing proteins, inhibiting ATPase[6] , distorting conidia and other fungal structures, as well as damaging DNA (Cuenca, 2006, pp. 33, 34; Valdés-Pérez *et al.*, 2016, p. 82).

From the structural point of view, the fungal cell wall plays a fundamental role for its vitality; it serves as a protective cover, provides cell morphology, facilitates both ion exchange and protein filtration, and participates in the metabolism and catabolism of complex nutrients. That is, it acts as a barrier, letting some substances pass through it whilst excluding others, so that part of those found on the surface can alter the physical and chemical properties of the membrane, preventing its normal functioning (Nazzaro *et al.*, 2019, pp. 10-11).

It should be noted that most fungi contain ergosterol as the main component of the membrane. Therefore, strategies that alter or block specific enzymes involved in ergosterol synthesis affect cell

[6] It has been referenced that eugenol inhibits ATPase, histidine decarboxylase, amylase and protease enzymes and ATPase inhibition promotes cell death (Valdés-Pérez, 2016, p. 82).

Family	Scientific name	Chemotypes	Microorganisms against which it has an effect	Mechanism of action
Lamiaceae	*Thymus vulgaris*	Carvacrol Timol Linalool	*Alternaria* *Aspergillus* *Candida* *Cryptococcus* *Fusarium* *Penicillium* *Rhizopus*	Affect membrane permeability. Damage to DNA. Loss of cellular elements. Denaturalisation of proteins. Metabolic stress. Disruption of vital functions. Irreversible damage. Growth inhibition.
Lamiaceae	*Satureja macrostema*	Carvacrol Timol Fenoles Flavonoides Limoneno Linalool	*Candida* *Colletotriuchum* *Fusarium* *Phytophthora* *Trichophyton* *Verticilum*	
Rutaceae	*Citrus Sinensis*	Limoneno Linalool Citronela Flavonoides	*Aspergillus* *Trichophyton*	
Rutaceae	*Citrus Paradisi*	Linalool Citronela Flavonoide Limoneno	*Aspergillus*	

FIGURE 2. Characteristics of the EO applied and the main mechanisms of action of the fungal strains studied (Table: Paulina Marlen Rivera Aguirre, 2024).

membrane permeability, causing irreversible damage by producing some metabolic stress and disrupting growth and vital functions, thus leading to growth inhibition (Trigos, 2007, pp. 92-94; Tang, Shao, Tang, & Zhou, 2018, p. 9).

EXPERIMENTAL DEVELOPMENT

Selection of waste material with microorganism attack

For this project, the study samples were selected based on the criteria of books belonging to the BPEJ collection, produced between 1800 and 1900, which had industrial paper as support, machine-made from wood species. In particular, those that have been

isolated from the rest due to different types of biological damage (microorganism, insect, rodent). Since the experimental process involved removing a page (Figure 3) and there were specimens in good condition, we opted for this temporality, thus avoiding the dissociation of elements in the case of an old book. On the other hand, the species that develop on the paper are the same, regardless of the age of the paper, and the use of alcohols can affect the proteins present in the constituent elements of an old book.



FIGURE 3. The selection of specimens chosen from the BPEJ (Photograph: Paulina Marlen Rivera Aguirre, 2023).

Although these materials were separated and stored under controlled humidity (55% ± 5%), illumination (cold LED), and temperature (21° ± 2° C), they were categorized as discarded material for academic purposes. These specimens had not been subjected to any previous intervention process and presented notorious microorganic infestation due to the fact that they had never been cleaned nor fumigated. After completing the selection, each specimen was assigned a name using the following acronyms:

- Guarda tipo carpeta, GTC
- Índice General Temático volumen IV, IGT
- *Mente fugaz* Núm. 9, MF
- *Geografía Universal Ilustrada*, GEO
- *Patrologiae Graecae Latine*, PGL

Process for obtaining samples and culturing microorganisms

The process began by checking that the microorganisms in the books were active; four samples were taken for each specimen in different areas that presented stains with characteristics that macroscopically differed from the rest of the support in color, cottony texture, and/or morphology. Acid-free, non-adhesive paper separators were placed to facilitate handling; sampling was performed in a sterile environment, using alcohol burners. A swab moistened with 0.5 ml of distilled water was used, applying the rolling method. In order to obtain a larger amount of sample, the swab was allowed to soak in the solution for 3 minutes. In order to reduce the residual humidity on the surface of the support, blotting paper and *monyl* mesh were placed both under and over the area to be sampled. The experiment was carried out, with the exception of obtaining the EO, in the Microbiology Laboratory of the Coordination of Conservation and Restoration at the BPEJ.

A cultivation method from the Dibico Malt Extract Agar® brand was used for this process, following the manufacturer's instructions. Distilled water was used, and everything was prepared and sterilised in an autoclave at 121° C (250° F) for 15 minutes. The pouring process into Petri dishes was carried out in a laminar flow hood, placing 25 ml in each dish.

The samples were all inoculated in the same way in Petri dishes that were placed for 7 days in a Felisa oven® at 28° C (82° F), in dark conditions and >60% relative humidity. This equipment allows for adjustments to the heating system and thermostat according to the needs of the study, making it possible to maintain the optimum temperature and humidity for any determined time needed to further the development of the microorganisms.

Identification of microorganisms

In order to determine the species of fungi present in the selected specimens, identification was carried out by a simple staining process using lactophenol blue; an acid dye that stains the cytoplasm and chitin present in the fungal cells and, at the same time, keeps the preparation moist to make appreciating its mycelial structure easier (López *et al.*, 2014, p. 16). The procedure consisted of selecting the colonies to be analyzed and cutting segments of adhesive tape (1 cm^2). Subsequently, a drop of the dye was placed on the slide, while the adhesive side of the tape was used to touch the top of the fungus removing the spores before immediately placing

it on the slide. This technique made it possible to determine the genus only, using a Zeigen® stereo microscope.

With this first analysis of microbial activity, mycelial growth was observed, in which four genera predominated, as shown in Figures 4 to 7: *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., and *Rhizopus* sp.

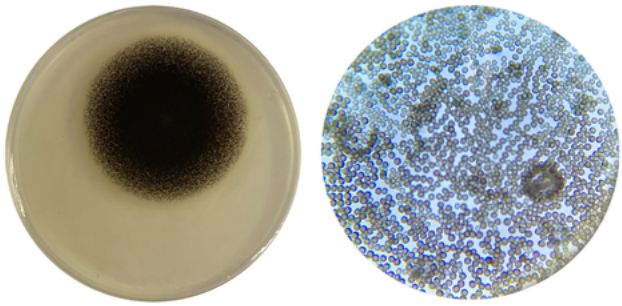


FIGURE 4. A) Brown color and a cottony, velvety texture are the main macroscopic characteristics of *Aspergillus* sp. B) Mycelial structure of *Aspergillus* sp. in the gemmation stage, observation at 100X (Photographs: Paulina Marlen Rivera Aguirre, 2023; courtesy of: Microbiology Laboratory, Coordination of Conservation and Restoration, BPEJ).

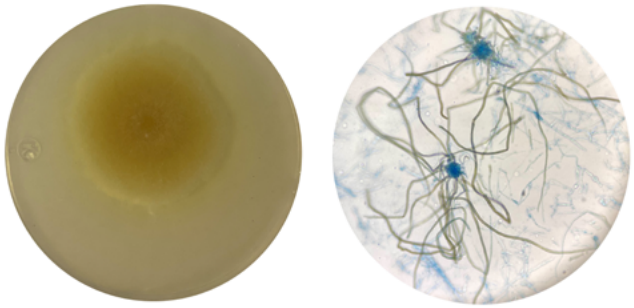


FIGURE 5. A) Velvet colony, with cream-colored radial folds that tend to darken to brownish tones. B) Branched sepals of *Cladosporium* sp., observation at 10X (Photographs: Paulina Marlen Rivera Aguirre, 2023; courtesy: Microbiology Laboratory, Coordination of Conservation and Restoration, BPEJ).

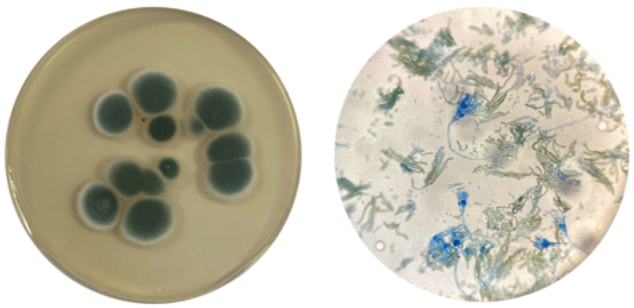


FIGURE 6. A) Velvety colonies with white or light-yellow mycelium framing the circumference; the fundamental growth surface is colored grayish green. B) Morphological structure that characterizes *Penicillium* sp., the brush-like conidiophore, observation performed at 40X (Photographs: Paulina Marlen Rivera Aguirre, 2023; courtesy: Microbiology Laboratory, Coordination of Conservation and Restoration, BPEJ).

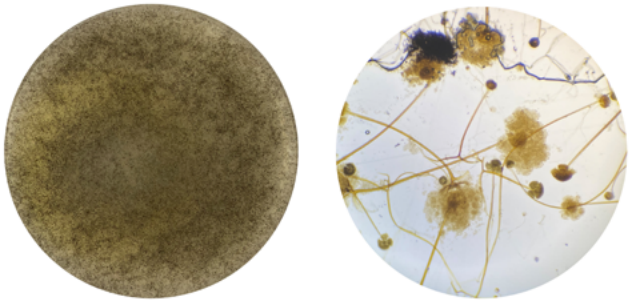


FIGURE 7. A) Fast-growing brown-colored colonies, with a cottony and downy aspect B) Mycelial structure of *Rhizopus* sp., with thread-like branched hyphae with spherical sporangiophores, observation at 10X (Photographs: Paulina Marlen Rivera Aguirre, 2023; courtesy: Microbiology Laboratory, Coordination of Conservation and Restoration, BPEJ).

Process for obtaining and preparing EO

The AE of *Thymus vulgaris* and *Satureja macrostema* were obtained at the facilities of the Centro Universitario de Ciencias Exactas e Ingenierías (CUCEI), specifically, at the Departamento de Madera, Celulosa y papel (DMCYP), belonging to the Universidad de Guadalajara (UDEG). The extraction process was carried out by hydrodistillation in a glass system consisting of a balloon flask, a condenser, a heating mantle, and a continuous Clevenger trap with a 10 ml collector. Once it was ground and washed, the plant material (50g) was weighed, and 200 ml of water was added. Heat was applied until the water boiled, with an extraction time of 4 hours. The isolated essential oil retained in the trap, with a volume of approximately 1 ml, was separated by decanting. Residual moisture was removed from the essential oil with anhydrous sodium sulphate.

The *Citrik*® Restoration disinfectant chosen was provided by the BPEJ. Based on the fact that it presents few specific studies regarding its action on a wide range of materials and fungal species, it is quite versatile in terms of the concentrations suitable for its application in relation to the types of materials and dissolved substances and the conditions of the environment in which it is applied, although this does not prevent it from being widely used in the field of restoration and in the BPEJ. For all these reasons, the decision was made to work with four concentrations: 2%, 5%, 7%, and 10%, dissolved in isopropyl alcohol brand Karel98® absolute and used by means of nebulization. Thus, the selected pages were divided into four each giving an overall of twenty samples to work with.

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Application of antifungal EO in fungicide test tubes

As for the application, it was carried out in a Marco Polo fumigation chamber model Ex-Libris® (CFE-110), which has a channel system, through a hose that allows the passage of very fine particles into its interior. Its design facilitates the monitoring of the appearance, temperature, and humidity of the interior during the process.

Subsequently, the specimens were introduced horizontally into the chamber, separated from each other by grilles. The EO and the commercial disinfectant *Citrik*® Restoration were diluted in isopropanol and applied by means of an Omron nebulizer®, model NE-C801LA. A total of 5 ml was used for each concentration. The nebulizer was chosen instead of the humidifier because it atomizes the liquid into fine particles, avoiding wetting the contact surface as well as the benefit of the isopropanol evaporating quickly without leaving residue.

At the end of each application, the test tubes rested in the chamber for 15 minutes with the fan on to allow the remaining residue to circulate in the environment. After this time, periodic sampling was carried out in the same areas for the concentrations: 2%:24 hours; 5%:24 and 48 hours and 15 days; 7%:24 and 48 hours and 15 days; and, finally, 10%:24 and 48 hours and 15 days (Figure 8), in order to obtain quantitative results on the possible inhibition of each substance. Inoculation was carried out in Petri dishes in a single direction in the form of a striation and incubated at 28° C (82° F) in a Felisa® brand oven for 7 days.

ANALYSIS OF RESULTS

The results obtained confirm that all oils have antifungal activity and vary according to the concentration applied and the type of microorganism involved. Regarding concentrations at 2%, *Aspergillus* sp. shows greater inhibition for *Thymus vulgaris*, compared to *Satureja macrostema* and *Citrik*® Restauración (Figure 9); however, the colony covers almost the entire surface of the Petri dish, although it is considerably reduced when increasing the concentration to 7% and 10% (Figures 13 and 15), with the presence of one to two colonies covering $^{1}/_{4}$ of the surface in the three substances (Figure 14).

Fungicidal substance	Concentration applied	Periodic sampling post-disinfection	Growth halo assessment
Thymus vulgaris	2%	24 hrs	7-day incubation (82°F)
	5%	24 hrs, 48 hrs, and 15 days	
	7%		
	10%		
Satureja macrostema	2%	24 hrs	
	5%	24 hrs, 48 hrs, and 15 days	
	7%		
	10%		
Citrik Restauración ®	2%	24 hrs	
	5%	24 hrs, 48 hrs, and 15 days	
	7%		
	10%		

FIGURE 8. Number of repetitions and experimentation levels (Table: Paulina Marlen Rivera Aguirre, 2024).

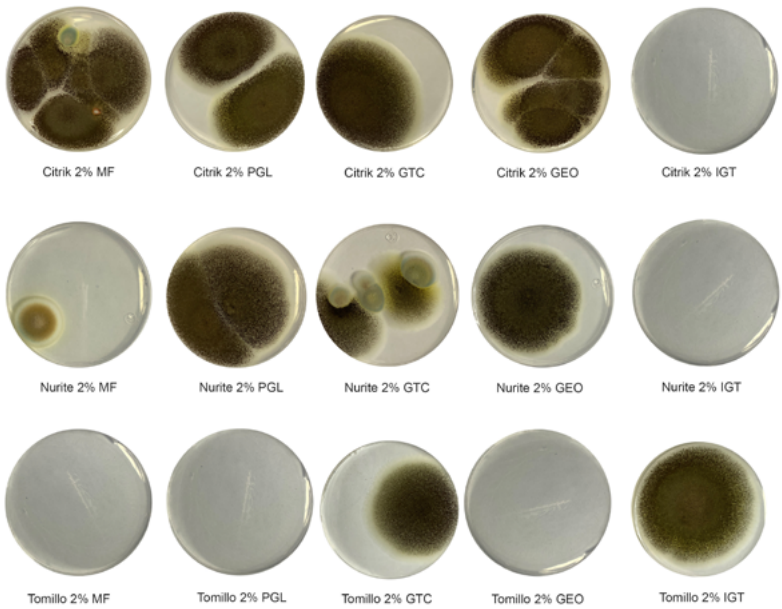


FIGURE 9. Results of concentration at 2%, 24 hrs, post application. Seven days of incubation (Photographs: Paulina Marlen Rivera Aguirre, 2023; courtesy: Microbiology Laboratory, Coordination of Conservation and Restoration, BPEJ).

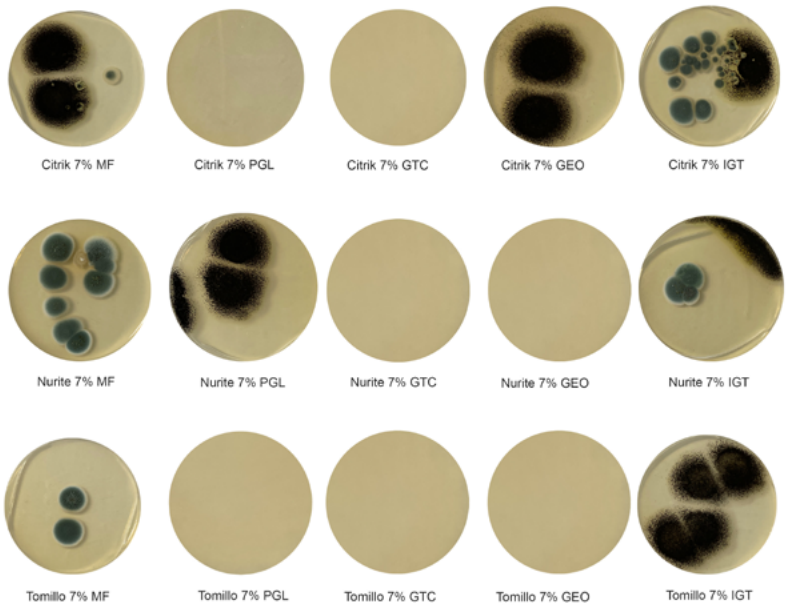


FIGURE 13. Concentration results at 7%, 15 days post application. Seven days of incubation (Photographs: Paulina Marlen Rivera Aguirre, 2023; courtesy: Microbiology Laboratory, Coordination of Conservation and Restoration, BPEJ).

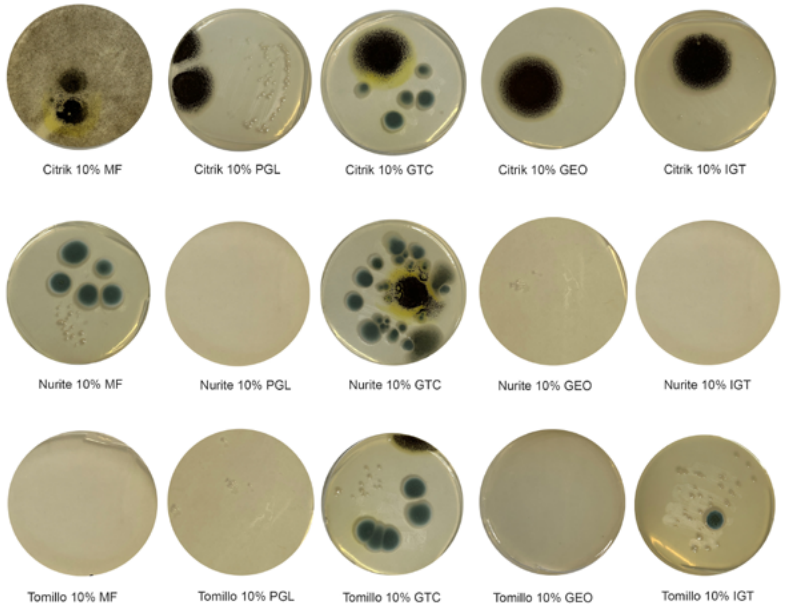


FIGURE 14. Concentration result at 10%, 24 hrs, post application. Seven days of incubation (Photographs: Paulina Marlen Rivera Aguirre, 2023; courtesy: Microbiology Laboratory, Coordination of Conservation and Restoration, BPEJ).

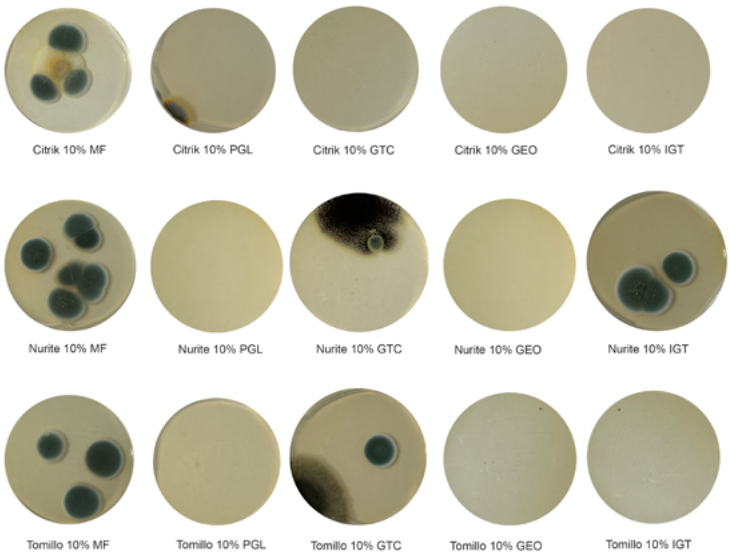


FIGURE 15. Concentration result at 10%, 15 days post application. Seven days of incubation (Photographs: Paulina Marlen Rivera Aguirre, 2023; courtesy: Microbiology Laboratory, Coordination of Conservation and Restoration, BPEJ).

In relation to the presence of *Cladosporium* sp., it covered ¼ of the surface and was completely inhibited at 7% with *Thymus vulgaris*, *Satureja macrostema* and *Citrik*® Restauración (Figure 10). On the other hand, *Penicillium* sp. shows a decrease in the halo as the concentration is raised for *Satureja macrostema* and *Citrik*® Restauración (Figures 11, 12, and 14); likewise, considerable inhibition is observed in the colony circumference for *Thymus vulgaris* (Figures 10, 12, and 13). As for *Rhizopus* sp. it does not show significant changes in relation to *Satureja macrostema* and *Citrik*® Restauración (Figures 10 and 14), unlike *Thymus vulgaris*, which shows total inhibition.

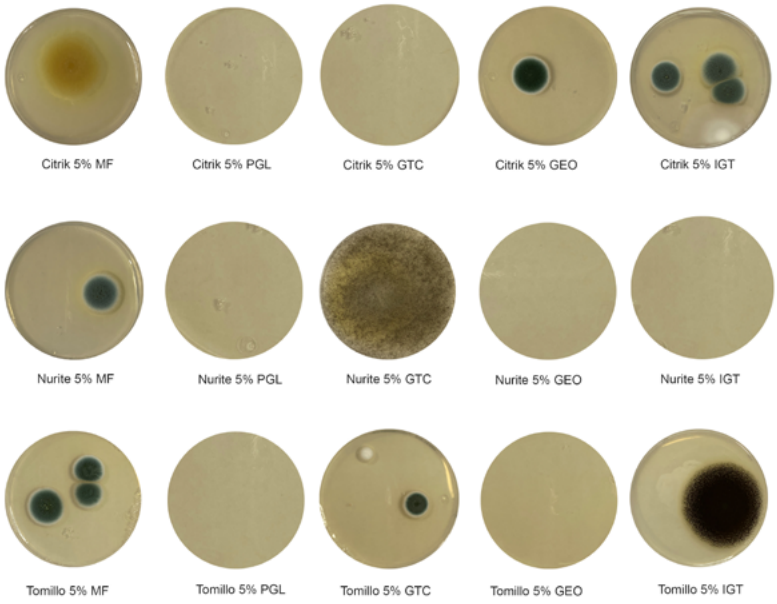


FIGURE 10. Results of concentration at 5%, 24 hrs, post application. Seven days of incubation (Photographs: Paulina Marlen Rivera Aguirre, 2023; courtesy: Microbiology Laboratory, Coordination of Conservation and Restoration, BPEJ).

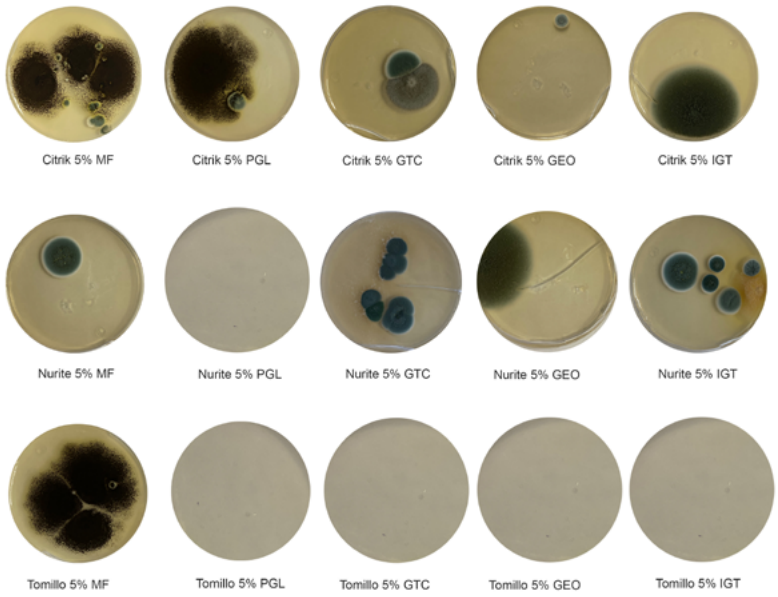


FIGURE 11. Concentration results at 5%, 15 days post application. Seven days of incubation (Photographs: Paulina Marlen Rivera Aguirre, 2023; courtesy: Microbiology Laboratory, Coordination of Conservation and Restoration, BPEJ).

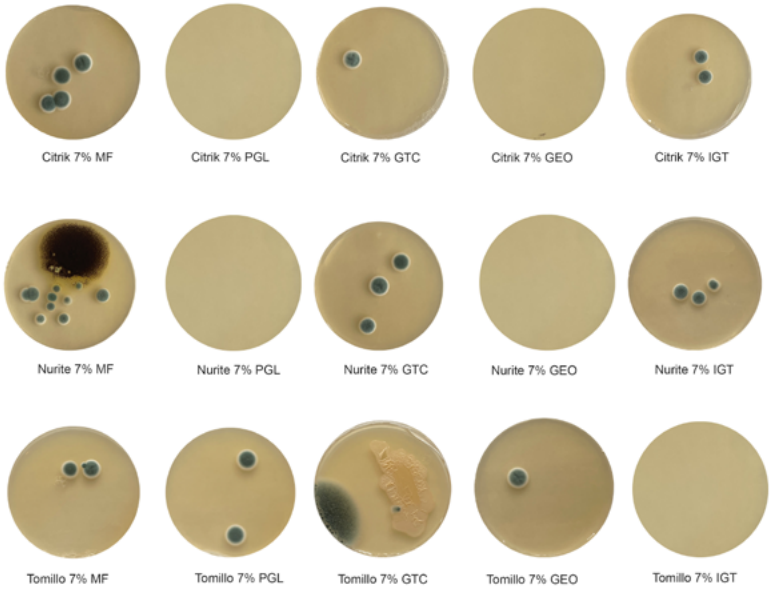


FIGURE 12. Results of concentration at 7%, 48 hrs, post application. Seven days of incubation (Photographs: Paulina Marlen Rivera Aguirre, 2023; courtesy: Microbiology Laboratory, Coordination of Conservation and Restoration, BPEJ).

Finally, with the three substances applied, *Cladosporium* sp. is the most susceptible fungus, since all the concentrations evaluated inhibit it, followed by *Aspergillus* sp., which, in terms of sensitivity, increases its effectiveness as the percentage increases. On the other hand, *Penicillium* sp. exhibits a notorious resistance, evidenced by the gradual decrease in the halo at higher concentrations.

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CONCLUSIONS

In the field of archival, library, and museum conservation, aromatic plants were historically used to poison the glue of books to both extinguish micro-organisms and to help repel insects. However, these methods were displaced by chemical products, and knowledge about them was transmitted mainly through practice and availability, rather than by a thorough analysis of their effectiveness.

There is little information regarding the use of natural products in the conservation of cultural heritage, few restoration professionals have mentioned their application in photography, wood, stone, or mural paintings. To mention a few, in 2011 the Cultural Heritage Institute of Spain (IPCE) developed a research project on natural extracts for the conservation of cultural property in organic supports as an alternative to conventional microbicides and insecticides on objects subject to biodeterioration, using species of the genus *Thymus* (Morales *et al.,* 2013, pp. 148-162).

Likewise, in 2016, the National Archive of the Republic of Cuba delved into the evaluation of the antifungal activity of clove EO, of pure odor, dissolved in ethanol at 70%. The paper components maintained their appearance in the presence of the EO without showing a significant affectation at the microscopic level, even after being subjected to 25 years of accelerated aging. However, at the macroscopic level it showed stains on the support according to the percentage of concentration, since at high concentrations the stains became darker (Valdés-Pérez *et al.* 2016, pp. 79-80).

Likewise, EO from *Organum vulgare, Thymus vulgaris*, and *Syzygium aromaticum* were used on six different genera of fungi, isolated from the wall paintings of the hermitage of Santa María de Arcos in Tricio, La Rioja, Spain. The antifungal capacity of EO was tested at 10% on the surface of a test tube with similar characteristics to the paintings (Rio, 2019, pp. 49-51).

In 2020 Cairo University (UC) in Egypt, evaluated the antimicrobial activity of five oils vapours (lavender, clove, anise, cinnamon, and thyme) on silver gelatine photographs, albumin, and chromogenic prints, it was shown that the tested EO were able to inhibit or slow down the growth of microorganisms; moreover, no color and brightness changes were presented on the study cases, until after being subjected to aging, although the change was minimal (Ali, 2020, pp. 81-85).

Meanwhile, at the Antonio Pasqualino International Puppet Museum of Palermo in Italy, Palla and collaborators also evaluated *Origanum vulgare* and *Thymus vulgaris* EO against fungal colonization and insect infestation on wooden sculptures, where their

efficacy was confirmed by decreasing the fungal inhibition halo in conjunction with oxygen reduction and so examining the use of EO for its repellent properties (Palla *et al.,* 2020, pp. 2-8).

Most evidently, the results we obtained confirm that the antimicrobial activity of EO varies according to the type of microorganism, the quantity and age of the infestation, the chemical composition and concentration of the oil, the characteristics of the constituent material and the degree of deterioration.

Although there is no universal treatment for the inhibition of fungal growth, it is important to highlight that natural products such as EO, are an alternative to reduce the toxicity of conventional biocides.

These findings show that more research is needed on alternatives to synthetic chemical biocides, not only in Mexico, but worldwide. Recently, Roberta Russo and Franco Palla reaffirm this view, in their analysis of the countries that have researched essential oils entitled *Aceites Esenciales Vegetales como Biocidas en Estrategias Sostenibles para la Conservación del Patrimonio Cultural* (Vegetable Essential Oils as Biocides in Sustainable Strategies for the Conservation of Cultural Heritage), in which they conclude that there is no uniformly accepted literature on the applications of EO nor for shared protocols within the academic community. Studies are more frequent in Europe (Italy, Portugal, Serbia, Malta, Poland, Greece, and Romania) than in other continents (America, Africa, and Asia) (Russo & Palla, 2023, p. 11).

Consequently, it is necessary to identify the colonizing organisms prior to the fumigation process, with macro and microscopic observations, before making decisions on the method of treatment. Each organism reacts differently to different concentrations and the chemical composition of the particular fungicide, the characteristics of the constituent material and the degree of deterioration present at the time of treatment.

It is worth noting that fungicides are generally designed to be selective in only targeting specific cells, so that they should always have some degree of impact even when full extermination is not achieved. By interrupting or altering vital functions, the fungicide prevents the fungus from colonising any further, thus allowing the biodeterioration of the substrate to be controlled, together with appropriate monitoring over the relative humidity, temperature, and lighting conditions in the environment where the micro-organism is growing.

Meanwhile, EO extracted from plants with established antifungal properties have, thanks to technological advancements, emerged

as viable options for more sustainable practices in conservation treatments. The versatility of these essential oils makes them ideal for the varied conditions and challenges that may face conservationists, and they carry the added bonus of connecting with the pieces on a heritage level since many of them are extracts that were once used in ancient medicinal practice, where knowledge about the healing properties within Mexico's unique plant biodiversity has been handed down through generations, and, which even spikes curiosity as to new lines for future research related to EO, microorganisms and their relationship with the environmental factors they are consistently exposed to. Furthermore, the more these eco-friendly methods are used the more studies inevitably follow, which should lead to new knowledge on the short-, medium- and long-term behaviour of the substrates with which they interact, further perfecting sustainable strategies in the area of conservation-restoration of cultural property.

Using essential oils for the fumigation process described here can be carried out in a single chamber, in the case of several volumes, or if in an enclosed space, it can also be done by means of manual nebulisation. The *Citrik*® Restauración and *Satureja macrostema* were found to be effective at concentrations higher than 5%, while *Thymus vulgaris* shows favourable results at 2% and above. As these products are of plant origin, they do not expire and the quantity required for their use is minimal, meaning they serve a long and useful life. Thus, from a multidisciplinary perspective, knowledge in the area of the microbiology of organisms and the understanding of the mechanisms of biodeterioration are determining factors in ensuring the conservation of cultural heritage, whether on paper or in other materials that constitute it.

In conclusion, the precise analysis of both the object and the conditions in which it is located —be it alteration, deterioration and/or context— opens up a dialogue for interdisciplinary and socio-cultural decision-making regarding the needs of the pieces requiring intervention. It is essential to be aware of the impact that the treatment methods applied in the area of conservation-restoration have or may have, not only on the piece being treated, but also on the immediate natural environment and any consequent potential risks to humans. At the same time, learning and applying eco-friendly methods promotes professional ethics aligned with the principles of sustainability and environmental responsibility.

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