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PLANT EXTRACTS AS GREEN POTENTIAL STRATEGIES TO CONTROL THE BIODETERIORATION OF CULTURAL HERITAGE

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Abstract

The biodeterioration of historic-artistic manufacts is related to several biological systems, including fungi and bacteria, whose metabolic activities and vegetative development have a direct consequence on the conservation of cultural assets. Generally, different chemical compounds are utilized as biocides in order to control biodeteriogens growth, but recently the attention has been focused on potential risks of their use towards human health (operators, visitors) and the environment. In order to develop alternative methods, various natural products have been tested, particularly to control the colonization by fungi and bacteria. In this study, antimicrobial activity of three different plant products, Tea tree essential oil, Calamintha nepeta and Allium sativum L. extracts, has been evaluated against Bacillus subtilis, Micrococcus luteus, Penicillium chrysogenum and *Aspergillus spp. (previously isolated from colonized artworks) through three different in vitro antimicrobial assays (micro-dilution in microtiter plates, well plates diffusion and agar disc diffusion method). The bioassays show a different microbial susceptibility to plant extracts, establishing for each bacteria and fungi the Minimum Inhibitory Concentration (MIC) and defining the diameter of the growth inhibition area. This result supports the data reported in literature and shows an important potential suggestion for the possible use in the control of biodeterioration of cultural heritage, safe both for human health and environment.*

Keywords: *Biodeteriogens; Antimicrobial activity; Plant products; Antimicrobial assays; Cultural assets*

Introduction

The beneficial effects of many plants, in particular their antimicrobial properties, are known since ancient time; in the last decade, many studies have been published in regards to screening the antimicrobial activity of various natural extracts, used to treat various infectious diseases, for the purpose of implementing their use in several fields including the cultural heritage protection. In order to control the biodeterioration phenomena, the natural antimicrobial compounds may represent a valid alternative to traditional biocides, which are generally toxic and not degradable, being persistent in the environment and causing contamination also in areas far from the site of application [1-13]. Several studies have been focused on the application of different plant extracts or essential oils, evaluating their

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antimicrobial activity against fungi and bacteria generally associated with the biodeterioration of cultural heritage [10, 14, 15]. The plant extracts act through different ways, as the regulation of intermediary metabolism, activation or blocking of enzymatic reactions, by direct effects on enzyme synthesis or alteration of membrane structures [16].

Antimicrobial activity of some essential oils, especially against microorganisms isolated from archives, libraries, and museums [10, 17, 18] has been recently evaluated for the biodeterioration control [19] and the information obtained so far are inadequate.

In the present study three plant products, *Tea Tree Oil* (essential oil), *Calamintha nepeta* and *Allium sativum* L. extracts, have been selected to test their antimicrobial activity against four microbial strains (*Bacillus subtilis*, *Micrococcus luteus*, *Penicillium chrysogenum*, *Aspergillus* spp.), previously isolated from colonized artifacts. The currently available screening methods for the detection of antimicrobial activity of natural products are the dilution and diffusion methods [15, 28]. The antimicrobial screening methods utilized to assess the antimicrobial activity of the three plant products were *microdilution in microtiter plates*, *well plate diffusion* and *agar disc diffusion* method.

Experimental

Plant materials and preparation of extracts

Tea Tree Oil (TTO) is the essential oil extracted from *Melaleuca alternifolia*, a native plant of Australia, widely used for its antimicrobial properties and often incorporated as an additional ingredient of multiple products [20].

The essential oil origin is not always clearly understood, since the name may refer to different products, based on the geographical location in which the plant is collected. The TTO 100% pure essence used in this experimentation is marketed by the company Esi®.

Calamintha nepeta is an aromatic perennial herb belonging to the *Labiatae* family, common in woods and the ruins of Western Europe and Asia. Modern medicine indicates the *C. nepeta* among the essential oils with antimicrobial highest value [21-23]. The *C. nepeta* was collected in Galati Mamertino (Messina, Italy) countryside during the summer season. The extract was obtained from *C. nepeta* leaves by steam distillation.

Allium sativum (garlic) is a species belonging to the *Allium* genus (family *Alliaceae*), distributed all over Europe, North America, Northern Africa and Asia. The *Allium* genus is rich in flavonoids, saponin, sapogenin and volatile sulphur compounds and their characteristic organoleptic properties derived from the presence of non-volatile flavour precursors, alk (en) y-L-cysteine sulfoxides [24]. Many sulphur compounds found in *Allium* species had antimicrobial [25], antiprotozoal, antioxidant, antihypertensive, hypolipidemic, hepatoprotective or antithrombotic properties [26]. The *C. nepeta* and *A. sativum* extracts were both obtained in the Laboratory of Natural Products at the Biological, Chemical and Pharmaceutical Science and Technology Department (STEBICEF), where the chemical compounds were characterized by Gas Chromatography-Mass Spectrometry (GC/MS).

Microorganisms and growing conditions

The tests were carried out using microbial strains isolated from different cultural objects and from museum environments, identified through microscopy and molecular biology investigations [27]. The bacterial and fungal colonies were *Bacillus subtilis*, *Micrococcus luteus* and *Penicillium chrysogenum*, *Aspergillus* spp., growth in nutrient broth for 24/36 hours at 30° C. The suspensions of the microbial cells were adjusted to a concentration of 1×10^6CFU (Colony Forming Units)/ml, referring to the McFarland scale.

Antimicrobial screening

The antimicrobial activity of natural extracts was determined using three different *in vitro* antibacterial and antifungal assays in order to develop the most appropriate method: *agar disc diffusion*, *well plates diffusion* and *microdilution in microtiter plates* [5, 28].

Agar disc diffusion. For the *agar disc diffusion* method [29] a paper disc (4mm in diameter) was placed onto the surface of Nutrient or Sabouraud agar plates (15mL in 90 mm Petri dish), wetted with 10µL of plant extracts (different concentrations = 100%, 50%, 25%, 12.5%). The plates culture medium has been previously seeded by microbial cells (concentration = 1×10^6CFU/mL) and incubated for 24/36h at 30 ± 1ºC, under aerobic conditions. After incubation, confluent microbial growth was observed and the diameter (mm) of growth inhibition areas was measured (sensible > 9mm, resistant < 9mm); precisely, each test was performed in triplicate. As controls, discs wetted with ethanol 70% or Benzalkonium chloride + chlorhexidine (0.2% (vol/vol) were included.

Well plate diffusion. The microbial inoculum (1×10^6CFU/mL) was uniformly spread using sterile cotton swab on Petri dish (Nutrient and Sabouraud agar), on which holes of 4 mm in diameter were punched aseptically [29]. 10µL aliquots (100%, 50%, 25%, 12.5%) of natural products were loaded to each well of the plate.

After 24/36h of incubation at 30 ± 1ºC in aerobic conditions, the plates were analysed in order to measure the diameter (mm) of growth inhibition areas. Each test was performed in triplicate.

Microdilution in microtiter plates. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) were assessed by the *microdilution method*, performed in 96-wells microtiter, distinguishing between biocide or biostatic action [30]. In each well, containing 30µL of plant extracts at different concentrations (100%, 50%, 25%, 12.5%) and liquid nutritive medium (Nutrient Broth), an equal volume of microbial suspension (1×10^6CFU/ml) was added. In order to facilitate the dispersion of the oil in the liquid medium, it was diluted with 1% of Tween 80 (not toxic for microbial cells). Aqueous solutions of the biocides, Benzalkonium chloride + Chlorhexidine (0.2%, vol/vol) and Nipagin (Methylparaben, 2.5mg/mL) were used as standard antimicrobial agents. Microbial growth after 24/36 h of incubation at 30°C was revealed by estimating the optical density at 500-600nm.

The MIC value was determined as the lowest concentration of the sample at which the tested microorganisms did not demonstrate any visible growth, after incubation time. The MBC and MFC were determined as the lowest concentration of antimicrobial when the number of colonies on sub-culture (antimicrobial-free media) indicated the 99.5% killing of the original inoculum [31].

Results and discussion

Microbial sensitivity to plant products

The *growth inhibition area* revealed by *well plate diffusion* (Table 1) and *agar diffusion disc* methods (Table 2) showed different antimicrobial effects against the four tested microbial strains; inhibition halos with distinct diameters were revealed in relation to the plant extracts.

Tea Tree Oil showed a positive activity (more than 9mm) against bacteria at maximum concentration (100%) and a moderate activity (between 6 and 9mm) at the other concentrations (50%, 25%) (Fig.1). The antimicrobial activity was higher against *Bacillus subtilis* (Fig. 1a) than *Micrococcus luteus* (Fig. 1b). *Tea tree Oil* had positive activity against *Penicillium chrysogenum* (Fig. 2) and no activity against *Aspergillus spp.*

Calamintha nepeta showed similar results to *Tea Tree Oil* for bacteria, but presented very low activity against fungal strains (Table 1 and 2).

A strong antimicrobial activity was performed by *Allium sativum* (Fig. 3). In particular, it exhibited the highest activity against all four strains at all concentrations tested. Bacterial inhibition (MIC) was evaluated by microdilution method (Table 3). Also in this case, there have been different responses: *Tea Tree Oil* and *Calamintha nepeta* extract showed biocide activity

against *M. luteus* and *B. subtilis* (Fig. 4). Moreover, *Allium sativum* had both biocide and biostatic activity against *M. luteus* and biocide activity against *B. subtilis* (Fig. 5).

Table 1. Well plate diffusion method. Average inhibition halo diameter (mm) for the different oils tested. Antimicrobial activity: Positive ≥ 9 mm; Moderate: 6 - 9 mm; Negative ≤ 6mm; total growth inhibition (*).

Essential oils (Scientific name)	Conc. (%)	Diameter of inhibition zone (mm) *Bacillus subtilis*	*Micrococcus luteus*	*Penicillium chrysogenum*	*Aspergillus spp.*
	100	15	7	8	2
Tea Tree Oil	50.0	10	6	4	0
	25.0	8	4	2	0
	12.5	4	4	2	0
	100	10	11	8	6
Calamintha nepeta L.	50.0	5	8	6	4
	25.0	5	6	4	2
	12.5	2	2	4	0
	100	15	*	20	4
Allium sativum	50.0	10	*	15	2
	25.0	10	*	8	2
	12.5	5	*	3	0

Table 2. *Agar disc diffusion method*. Average inhibition halo diameter (mm) for the for plant extracts tested. Antimicrobial activity: Positive ≥ 9 mm; Moderate: 6 - 9 mm; Negative ≤ 6mm; total growth inhibition (*).

Essential oils (Scientific name)	Conc. (%)	Diameter of inhibition zone (mm) *Bacillus subtilis*	*Micrococcus luteus*	*Penicillium chrysogenum*	*Aspergillus spp.*
	100	36	31	41	0
Tea Tree Oil	50.0	22	21	24	0
	25.0	21	14	21	0
	12.5	16	10	21	0
	100	15	12	10	9
Calamintha nepeta L.	50.0	10	8	7	4
	25.0	7	6	4	2
	12.5	2	0	4	0
	100	20	9	*	*
Allium sativum	50.0	11	4	*	*
	25.0	11	3	*	*
	12.5	6	3	*	*

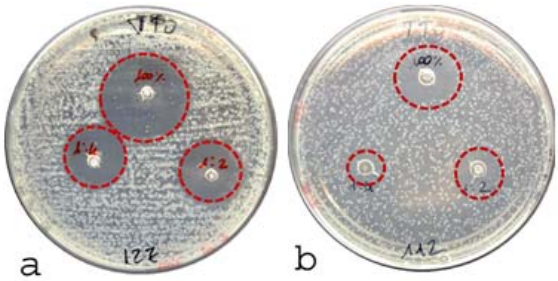

Fig. 1. *Agar well diffusion method*. Different inhibition halo diameters (red circles) for Tea tree Oil (concentrations 100%, 50%, 25%) referred to *Bacillus subtilis* (a) and *Micrococcus luteus* (b).

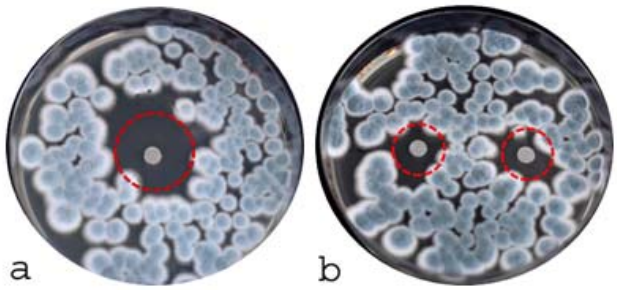

Fig. 2. *Agar disc diffusion method*. Different inhibition halo diameters (red circles) for Tea tree Oil at 100% (a), 50% and 25% (b) referred to *Penicillium chrysogenum*.

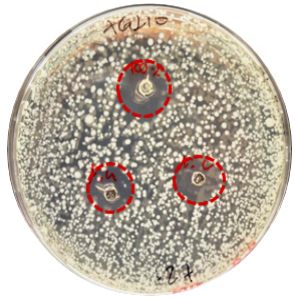

Fig. 3. *Agar well diffusion method*. Different inhibition halo diameters (red circles) for *Allium sativum* at 100%, 50% and 25% referred to *Bacillus subtilis*.

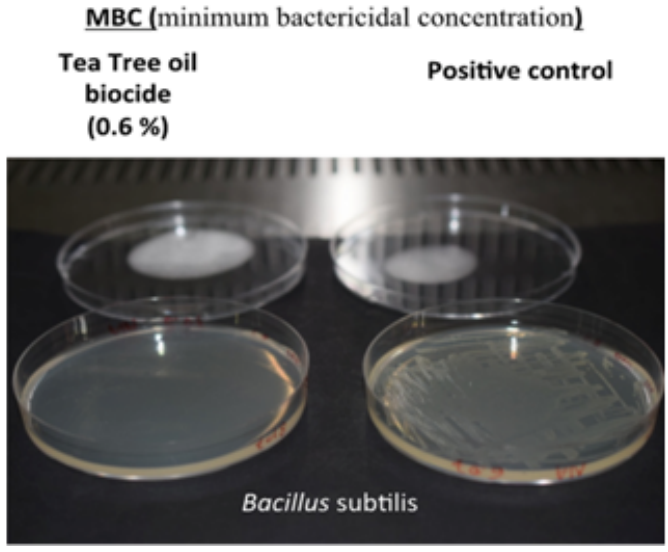

Fig. 4. Minimum bactericidal concentration (MBC). Sub-cultivations into fresh medium lacking Tea tree Oil. The lowest concentration with no visible growth (0.6%) is showed. Positive control: inoculum of *Bacillus subtilis* (10^6 CFU/ml).

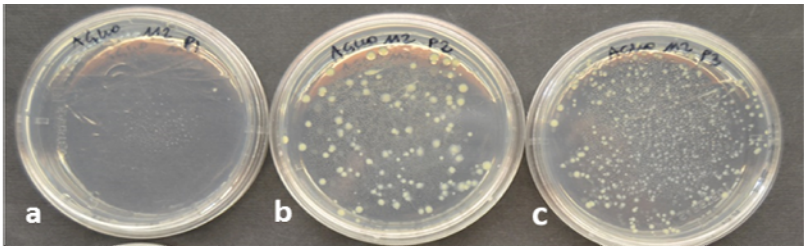

Fig. 5. Minimum bactericidal concentration (MBC). Serial sub-cultivations into fresh medium lacking *Allium sativum* Biocide activity (a); biostatic activity (b, c).

These variable results could be due to the oils solubility or to the different susceptibility of the microbes to the substances, because all microbes present differences in the cell wall structure, lipid and protein composition of the cytoplasmic membrane, as well as in specific physiological processes [10, 32].

In this work, we also compared the sensibility between the performed tests: all three bioassays have given positive and buyable results, but *agar diffusion disc* method has been more easy to use; the disc will allow a better distribution of the extract on the plate, instead, the *well diffusion* method showed a better visualization of the inhibition halo.

Table 3. Minimum Bactericidal Concentration (%), biocide or biostatic action of oils tested.

Oil extracts	*M. luteus*	*B.subtilis*
Tea Tree Oil	Biocide (0.6%)	Biocide (0.6%)
Calamintha nepeta L.	Biocide (1.56%)	Biocide (1.56%)
Allium sativum	Biocide (100%) Biostatic (50% - 25%)	Biocide (100%)

Conclusions

Recently, the antimicrobial activity of plant extracts has been recognized in cultural heritage field but few investigations were carried out on the application of these extracts.

In this study, antimicrobial activity of three plant products *Tea Tree Oil*, *Calamintha nepeta* and *Allium sativum* L. was tested through by a*gar diffusion disc*, *well plate diffusion*, and *microdilution* methods.

The results obtained by assaying the natural extracts confirm the data reported in literature about the broad-spectrum antimicrobial activity. For the first time, we highlighted the possibility to apply *C. nepeta* extracts as antimicrobial on artifacts, which studies are ongoing.

The results allow us to hypothesize the use of these plant products as natural biocides in the control of biodeterioration of cultural assets.

More studies are needed to evaluate their permanence and durability on artifacts surfaces if stored/exposed in both open space and indoor environments. Moreover, we also focused our attention on the innocuousness of these plant extracts in respecting of the human health and the environment, according to modern biorestoration procedure.

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