Journal of Basic & Applied Sciences, 2022, *18*, 119-125

Published by SET Publisher

Journal of Basic & Applied Sciences

ISSN (online): 1927-5129

Plant Products as Biocides for Conservation of Cultural Asset Sustainable for Human and Environmental Health

Franco Palla*

University of Palermo, Laboratory of Biology and Biotechnology for Cultural Heritage, Department STEBICEF, Via Archirafi 38, 90123 Palermo, Italy

Article Info:

Keywords:
Biodeterioration,
plant extract,
green biocides,
molecular investigation,
cultural heritage.

Timeline:
Received: November 08, 2022
Accepted: December 10, 2022
Published: December 19, 2022

Citation: Palla F. Plant products as biocides for conservation of cultural asset sustainable for human and environmental health. J Basic Appl Sci 2022; 18: 119-125.

Abstract:

In the last decades plants products are increasingly used also in defining innovative procedures for sustainable conservation of cultural heritage. Particularly, essential oils or hydro-alcoholic extracts have been fruitful applied to contrast microbial colonization on organic and inorganic artworks or insect infestation (*Anobidae*) having regard to the repellent action.

In this paper, extracts from Asteraceae and Lamiaceae plant families are utilized to counteract widespread microbial colonization (bacteria, cyanobacteria, fungi) due to their antimicrobial activity. In order to define the adequate concentration correlating it to microbial species detected, the antimicrobial activity of natural products is preliminary *in vitro* assayed by Agar Disc and Well Plate Diffusion methods. Moreover, an integrated approach based on morphological analysis (optical, scanning and confocal electron microscopy), *in vitro* cultures (Nutrient or Sabouraud agar) and molecular investigation (genomic microbial DNA extraction, sequencing and sequence analysis) are routinely performed for the identification of largest number of microbial taxa.

Besides, to enhance the antimicrobial activity different protocols have been performed, such as for *Thymus vulgaris* products directly applied on wooden sculpture surface as hydro-alcoholic extract, followed by exposure, in a dedicated chamber, to the volatile compound of the corresponding Essential Oil. In other case studies, the exposure to volatile compounds of *Crithmum maritmum*, *Inula critmoides*, *Thymus vulgaris* and *Origanum vulgare* essential oils was performed under controlled *vacuum* conditions, comparing the effects to environmental condition exposure. These studies confirm the possible use of plant extracts in replacing synthetic chemical biocides, in full respect of human health and environment sustainability.

DOI: https://doi.org/10.29169/1927-5129.2022.18.12

*Corresponding Author
Tel: +39 91 23891224
E-mail: franco.palla@unipa.it

INTRODUCTION

Extracts from aromatic plants, spontaneous in the Mediterranean basin and currently cultivated all over the world, are used to contrast microbial colonization since ancient times [1-4]. Essential oil (EO) and hydro-alcoholic extract (HE) achieve an antimicrobial action through different ways, acting on cellular metabolism as well as on enzymatic reactions or synthesis, amending some membrane structures [5-7]. Several studies support the hypothesis that natural products can replace the synthetic chemical biocides, that as we know are toxic and able to persist for a long time in the environment, also contaminating areas far from the application site [8-11].

This paper reports that extracts from two different plant families, such as Lamiaceae (*Origanum vulgare* L. and *Thymus vulgaris* L.) and Asteraceae (*Crithmum maritmum* L. and *Inula crithmoides* L.), showed a good activity in contrast of microbial colonization, in conservation of cultural heritage protocols. These plants are rich in phenolic monoterpenes with a strong antimicrobial and antioxidant activities. Gas chromatographic analysis reveal several bioactive molecules such as carvacrol and thymol, beside cineol and thymol methilester, *p*-cymene and terpinol, borneol, geraniol, linalool alcohols, acetate and linalyl esters. Particularly, essential oils of *O. vulgare* and *T. vulgaris* were successful applied to counteract fungal colonization (*Aspergillus flavus*, *Aspergillus niger*, *Penicillium chrysogenum*) or insect infestation (*Anobium punctatum*) on wooden artworks [12-14], as well as to affect the development and vitality of a complex biofilm revealed under the tiles of the mosaic floor in the Solunto Archaeological Park, Sicily [15].

Furthermore, *I. crithmoides* EO shows antibacterial activity *vs Georgenia* sp., *Ornthinibacillus* sp. and *Streptomyces* sp. colonies, spread over the surface of leather artwork [16]. Concerning *C. maritmum* EO a reduced antibacterial activity has been revealed compare to *T. vulgaris,* although higher then Benzalkonium chloride, a broad-spectrum biocide frequently used in conservation procedure [17].

In relationship to biological colonization and artwork constitutive material, EO solution has been applied directly on a biofilm [15] or exposing microbial colonies to EO volatile compounds, in *ad hoc* exposure-chamber assembled by using a heat-sealable barrier film or Plexiglas foils [12, 14, 17].

Before application on artworks, key points are the evaluation of EO antimicrobial activity correlating it to the EO concentration and the microbial species identified, performing *in vitro* by Agar Disc Diffusion and Well Plate Diffusion methods [18, 19].

In order to define adequate strategies, the largest number of bacterial, fungal, cyanobacterial taxa must be identified, using an interdisciplinary approach [20, 21] based on morphological analysis (Optical, Scanning and Confocal Electron Microscopy observation), *in vitro* cultures (Nutrient or Sabouraud agar) and molecular investigation (genomic microbial DNA extraction, *in vitro* amplification of target sequences by PCR technology, amplicon sequencing and sequence analysis).

Particularly for *T. vulgaris* EO, in order to implementing its antimicrobial activity, two different strategies have been defined. In one case (ethno-anthropological wooden sculpture), the exposure to EO volatile compounds was preceded by the direct application of the corresponding hydro-alcoholic extract (HA) onto artwork surface, counteracting a spread microbial colonization related to *Aspergillus* sp., *Streptomyces* sp., *Micrococcus* sp. [22]. In the other, the exposure has been performed under vacuum conditions, observing an evident falling in bacterial (*Bacillus*, *Georgenia*, *Ornithinibacillus*, *Streptomyces* genera) colonization [17].

The results of these studies provide useful information on use of aromatic plants extracts as a valid alternative to synthetic chemical biocides, defining conservation protocols respectful of cultural heritage, operator and environment health.

EXPERIMENTAL SETUP

Microbial Taxa Identification by an Interdisciplinary Approach

The identification of microbial systems colonizing works of art surface was performed using an integrated approach, routinely used in our laboratory [23, 24] and summarized in Figure **1**: *i*) sampling, by sterile swabs (microbial colonies) or sterile scalpel (biofilm fragments); *ii*) *in vitro* culture, on Nutrient or Sabouraud agar (Difco), incubating for 18-48 hours at 30°C; *iii*) morphological analysis of bacterial colony or fungal conidia - conidiophores (Lugol iodine staining) by Optical and Scanning Electron Microscopy, or algae and cyanobacteria in complex biofilm by Confocal

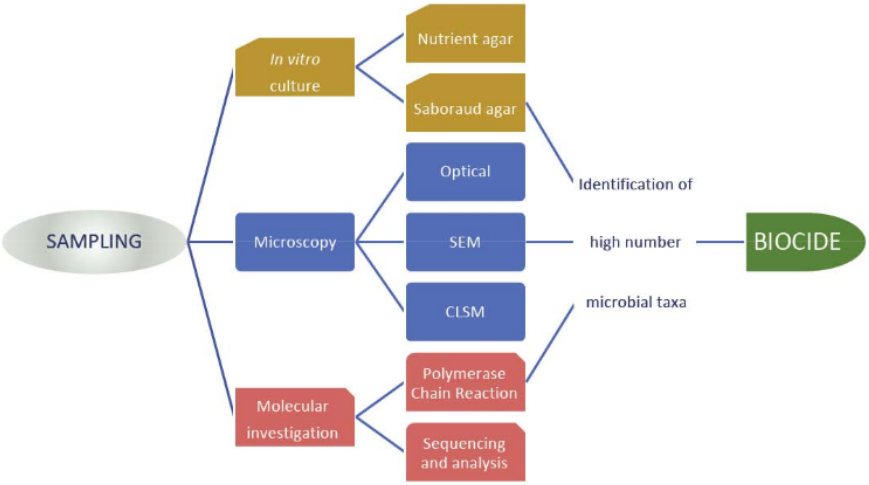

Figure 1: Scheme of interdisciplinary approach, allows the acquisition of peculiar information for choosing the adequate biocide.

Laser Scanning Microscopy; *iv*) molecular investigation, consisting of amplifying rDNA target sequences specific for bacteria (16S gene, ITS) of fungi (ITS1-ITS2 regions) by Polymerase Chain Reaction (PCR), amplicons sequencing (MWG-Eurofins, Germany) and the nucleotide sequences analysis (BLAST platform; EMBL-Germany, NIH-USA nucleotide databases). This interdisciplinary is finalized on revealing the high number of microbial taxa, colonizing the artwork, in order to utilize the appropriate biocidal approach.

Concerning microscopy analysis, fungal reproductive structure, can be revealed by Optical (after Lugol's staining) or Scanning Electron Microscopy (coating with gold - microparticles by Agar Auto Sputter), as shown in Figures **2** and **3**, for *Penicillium* and *Aspergillum* spp. respectively [25, 26].

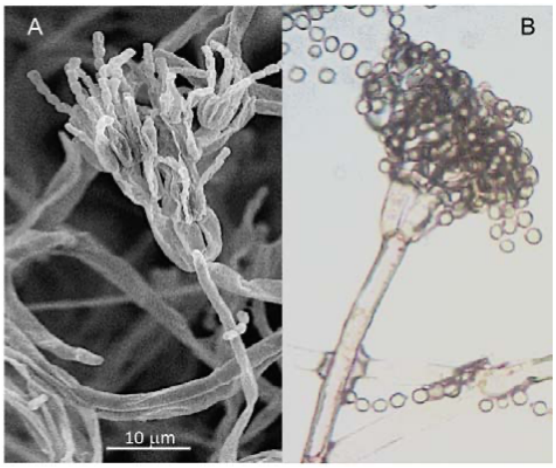

Figure 2: *Penicillium* sp. hyphae and conidia: **A**) SEM micrograph (Leica LEO 400 – 40x); **B**) Optical Microcopy (Leica 40X).

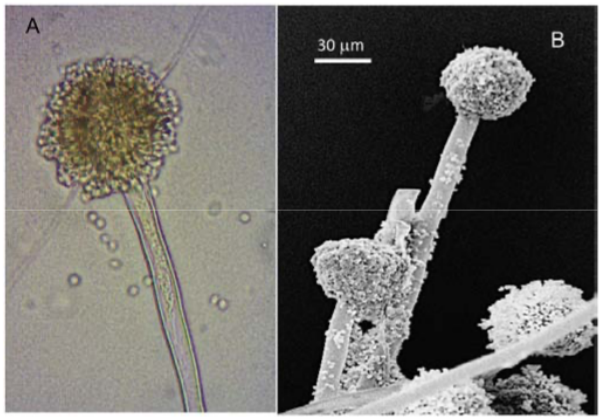

Figure 3: *Aspergillus* sp. structures: **A**) Optical Microcopy (Leica - 40X); **B**) SEM micrograph (Leica LEO-400 – 40x).

Confocal laser scanning microscopy (CLSM), a high-resolution optical imaging technique, can also be utilized to reveal the occurrence of microorganisms that naturally emit fluorescence, since they have pigments such as chlorophyll, carotenoid and phycobilin-proteins [27]; as shown in Figure **4**, CLSM allow the revealing of cyanobacteria colonies in the biofilm fragment sampled on marble artifact surface.

In order to complete the microbiological survey, molecular biology techniques allow the identification of the higher number of microbial taxa colonizing the cultural asset. The molecular investigation starts with the extraction of microbial genomic DNA from isolated colonies or directly from biofilm fragments, utilizing it as template in PCR reactions, for *in vitro* selective

amplification of specific target sequences. PCR products, resolved by gel electrophoresis, Figure **5**, corresponding to portion of bacterial 16S gene and ITS spacer of rDNA, with fragments of 150 to 400 bp in length.

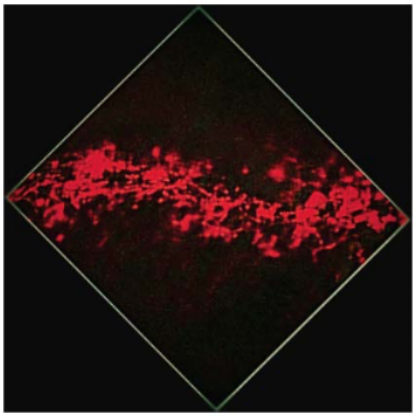

Figure 4: CLSM micrograph, auto-fluorescent microbial systems revealed by Olympus FV 300 – (Argon/Elio lasers = λ=488/543nm) – 40x magnitude.

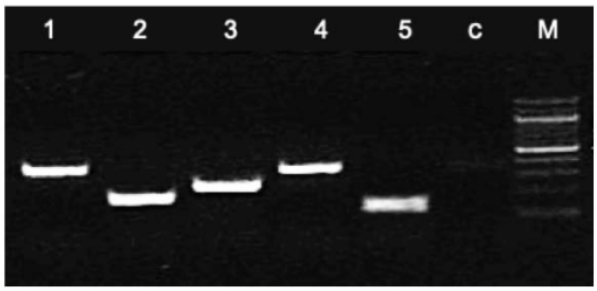

Figure 5: PCR products resolved on 2% agarose gel (lanes 1-5), Control reaction without DNA template (line c) and 100 bp DNA ladder (line M).

The nucleotide sequence of DNA amplicons was performed by Eurofins MWG Operon sequencing service and sequences comparison performed by BLAST platform [29].

Essential Oil and Hydro-Alcoholic Extract

Main chemical components of essential oils utilized in these studies were identified by GC–MS [12, 16, 17, 29-31] and summarized in Table **1**. Instead, *T. vulgaris* Hydro-Alcoholic extract has been assessed, according to the European Council Pharmacopoeia (carvacrol = 0.0010%; thymol= 0.12%; camphor, eucalyptol <0.0010%), by EPO Srl (Milano) that supplied the extract [32].

Evaluation of Antimicrobial Activity

Liquid cultures (16-48 hours of incubation at 30°C) were made using single bacterial (*Bacillus* sp., *Georgenia* sp., *Micrococcus* sp., *Ornithinibacillus* sp., *Streptomyces* sp.) or fungal (*Aspergillus flavus*, *A. niger*) colonies isolated on Nutrient and Sabouraud agar respectively.

Aliquots of each liquid cultures were utilized in growth-inhibition assays, specifically Agar Disc Diffusion (ADD) and Well Plate Diffusion (WPD) methods [15, 17, 22].

In the ADD test, a sterile paper disk (6 mm diameter, Dutscher papier, FR) is placed in the center of 90 mm culture agar plate and soaked with essential oil solution (10 µl of 50%, 25%, 12.5%). In the WPD assay, a hole of 4 mm in diameter is aseptically drilled in the center of 90 mm agar plate, filled in with 10 µl essential oil solution (50%, 25%, 12.5%). After incubation at 30°C, for 16-48 hours, confluent microbial growth is observed on the agar surface except in inhibition-halo area, whose diameter (mm) is related to the antimicrobial activity of EO components.

In parallel, specific control tests are performed, in which the paper disks are soaked with 3% (vol/vol) of Benzalkonium-chloride (commercial broad-spectrum biocide) or 70% Ethanol.

The antimicrobial activity is measured by the diameter of inhibition halo (ih), defining the microbial strain as:

Table 1: Main Chemical Compounds of EOs

Plant specie	Compounds (%)	Reference
I. crithmoides L.	*β*-Myrcene (13.7), *p*-cymene (11.7), Thymol acetate (14.4), Thymol (2.6)	[16]
O. vulgare L.	Thymol (27.18), *p*-cymene (18.97), carvacrol (4.04)	[12, 15]
T. capitata (L.) Cav.	Carvacrol (73.5), γ-Terpinene (6.9), *p*-cymene (4.3)	[25]
T. capitatus H. et L.	γ-Terpinene (34.4), *p*-cymene (22.8), carvacrol (14.2)	[24]
T. mastichina L.	1,8-Cineole (55.7), canphor (13.3), borneol (7.2), *a*-cymene (4.78),	[27]
T.vulgaris L.	Carvacrol (64.96), *p*-cymene (11.29), thymol (8.25)	[15, 17, 21]

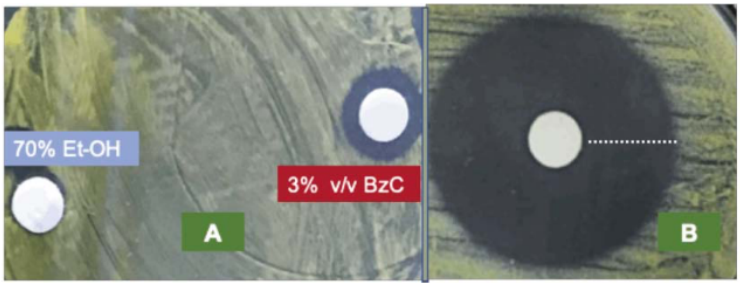

Figure 6: Agar Diffusion Disc assay vs. *Streptomyces* sp.: **A**) Control test performed with 70% Et-OH and 3% (v/v) Benzalkonium Chloride (BzC); **B**) the paper disk has been moistened with 25% *T. vulgaris* EO solution.

Inhibition halo is barely visible for Et-OH, just 2 mm for BzC and 14 mm for EO solution.

sensible = ih > 9 mm, resistant = ih < 6 mm, replicating each assay twice. Specifically, inhibition halo (14 mm) is clearly recognizable for the 25% *T. vulgaris* EO solution, significantly reduces (2 mm) for BzC solution (3% v/v) and hardly to detect for the 70% Et-OH solutions.

Application and Exposure to Plant Extracts

Generally essential oils are safely used in vapor phase, avoiding the direct contact with the artworks surface, performing the exposure in specific structure, such as desiccators cabinet (glass chamber) or *ad hoc* assembled clean-chambers (Gas-barrier thermo-sealed film, Plexiglas foils) and recently for improving the indoor air quality in dedicated cultural heritage environments [12, 14, 17, 33-35].

In a peculiar case study, Mali kapok wood (*Ceiba pentrada* L. Gaertn) sculpture, the exposure to EO vapor and direct application of related HA solution, has been possible using *Thymus vulgaris* extracts, since any finishing treatment was not revealed on sculpture surface [22]. The brown and smooth surface, as tradition of puppet theatre in Africa, is attributable to repeated treatments with hot spatulas [36].

As shown in Figure **7**, on the wooden sculpture surface a spread microbial colonization has clearly revealed and removed by direct application of the Hydro-Alcoholic extract, completing the treatment with the exposure to volatile compounds of EO.

The high microbial load revealed before has been deeply reduced, becoming to zero after 3 weeks of treatment. Microbial monitoring performed for one year after the re-exposition of the artifact in the museum environment, did not highlight potentially dangerous increases in microbial load.

Furthermore, to evaluate possible variations in the color of wood surface, before and after HA extract application, colorimetric measurements (PCE - CSM 7, portable colorimeter) were performed on specific areas of wood surface; not perceptible variation in the three parameters (L*, a*, b*) were detected [22].

RESULTS AND DISCUSSION

In recent decades, the request of innovative protocols for sustainable conservation of cultural assets has become ever greater and more focused on human and environmental health respect. Several aromatic plants ca be considered as source of bioactive compounds, representing useable molecules reduce both environmental pollution and toxicity to humans [8-11].

Although plant extracts can be considered antimicrobial agents, acting on microbial cells by several

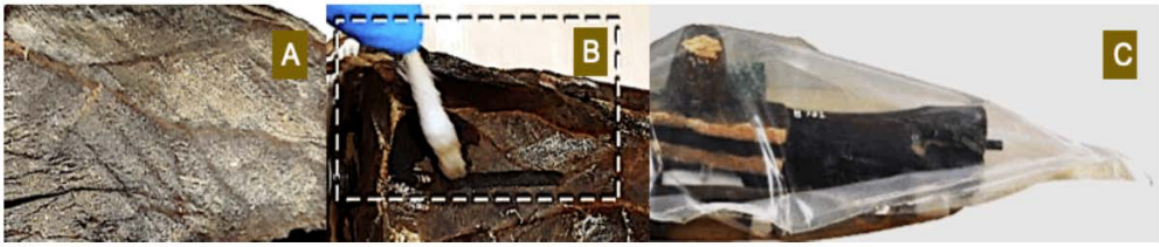

Figure 7: Cooperative actions strategy using *T. vulgaris* extracts. The whitish microbial patina on wooden surface (**A**) is removed by the HA solution (**B**) and subsequently exposed to EO volatile compounds in a dedicated clean-chamber (**C**).

mechanisms, also in synergic way, the avoiding of direct contact on artwork surface must be due; arbitrary alterations of pigments or varnishing surface layers can be induced.

The peculiar executive technique of Mali wooden sculpture, allows the combined use of *T. vulgaris* essential oil and hydro-alcoholic solutions, setting up a green strategy to counteract a spread microbial colonization on artwork surface, and the protection from re-colonization seems to be active after one year. Gas-chromatography analysis showed that carvacrol and thymol are the main compounds in both EO and HA solutions, and to these compounds is main attributable the antimicrobial activity [37, 38].

CONCLUSION

Considering the close connection between historical–artistic assets, promotion of culture and valorization of cultural heritage, in the perspective of transmission to the next generations, technical scientific research must be improved increasing the interest towards green approaches. The integrated approach showed in this paper allow to minimizing the sample amount needed for understanding the complexity of microbial communities colonizing both organic and inorganic substrates, also revealing unculturable species.

A complete identification of microbial taxa, able to induce the artifact biodeterioration, is strictly related to biocide choose.

This paper also suggests innovative protocols, based on green conservation strategy, respectful of both operators and environment, showing that plant bioactive compounds, such as essential oils and hydro-alcoholic extract, can certainly replace synthetic chemical biocides.

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