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Environmentally friendly products from plants in the control of biodeteriogen agents

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ABSTRACT

Since ancient times, extracts and essential oils from plants "vegetal biocides" have been used as antimicrobials in different fields. Using environmentally friendly products is presented as a viable solution that shows advantages from the environmental, economic and ecological standpoint that reduced use of toxic chemicals and pollutants to the environment. The aim of this paper is to present the studies that were used vegetal biocides for the control of microorganisms involved in biodeterioration and that pollute the environment of archives and libraries, and cause human health problems. The "vegetal biocides" were obtained from various plants harvested from natural habitat of La Plata, Argentina and Havana, Cuba. The biocidal activity was studied using the agar diffusion method. Vegetal biocides were analyzed by gas chromatography coupled to mass spectroscopy (GC/MS). The antimicrobial activity is due to sesquiterpenes, triterpenoids, flavonoids, sterol, phenols, i.a., and showed moderate effectiveness and/or positive in most vegetal biocides tested.

KEYWORDS

Essential oils;
Antimicrobial activity;
Biodeterioration;
Plant extracts;
Microorganisms;
Plants.

INTRODUCTION

Cultural heritage is constantly exposed to environmental influence[1, 2]. Physical, chemical and biological materials interact with promoting changes in structure and composition. Alterations caused by biological factors are called biodeterioration[3]. The intensity of deterioration is related to material composition, environmental conditions and associated microorganisms.

Biodeterioration affects not only aesthetic of the heritage assets but also can produce the degradation of them, causing material and economic losses. For example, the documents in libraries and archives have the enormous responsibility of safeguarding the graphic memory of the Nation for future generations, hence the importance of their protection and preservation. On the other hand, many microorganisms in archives and libraries cause damage to human health. *Aspergillus* spp.

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is one of the fungi of clinical interest because it has species that are capable of causing a lot of damages to people, such as Type I allergies (immediate hypersensitivity or allergic rhinitis followed by asthma attacks), sinusitis, otitis, keratitis and may lead to severe aspergillosis[4,5].

Chemicals that have traditionally been used as antimicrobials to prevent biodeterioration of cultural heritage, are generally pollute the environment and affect human health and cause changes in the material in which they are applied.

The application of environmentally friendly products from plants (extracts and essential oils) in the control of microorganisms involved in biodeterioration of cultural heritage is presented as a viable solution that shows advantages from the environmental, economic and ecological standpoint that reduced use of toxic chemicals and pollutants to the environment. The antimicrobial activity is due to the presence of phenolic compounds, terpenes, aliphatic alcohols, aldehydes, ketones, i.a.[6,7]. These compounds may act as intermediary metabolism regulators, activate or block enzymatic reactions directly affect enzymatic synthesis or alter membrane structures.

Since ancient times antimicrobial activity of extracts and essential oils of plants are known and, in recent years, there has been renewed interest of scientists from the use of these natural substances. However, among the many studies, few mention their use in the field of conservation of cultural property[8-10]. The aim of this paper is to present the studies that used biocides from plants for control of microorganisms involved in biodeterioration processes of materials library and archive.

EXPERIMENTAL

Obtention of extracts and essential oils from plants

The following plants harvested from their natural habitat were used: *Allium sativum L.* (garlic), *Arctium lappa L.* (burdock), *Artemisia sp.* (sagebrush), *Baccharis crispa* Pers (carqueja), *Camellia sinensis* (L.) Kuntze (tea), *Centaurea cyanus* L. (centaurea), *Cestrum nocturnum* L. (lady of night), *Cichorium intybus* L. (chicory), *Citrus sinensis* Osbeck (orange), *Conyza bonariensis* (L.) Cronquist (coniza), *Eucalyptus citriodora* Hook (eucalyptus), *Ilex paraguayensis* A-St. Hill (yerba mate), *Laurus nobilis* L. (laurel), *Medicago sativa* L. (alfalfa), *Mentha piperita* L. (peppermint), *Mentha arvensis* L. (mint), *Nerium oleander* L. (laurel rosa), *Origanum* x applii (origanum), *Origanum vulgare* (origanum), *Petroselinum crispum* (Mill.) Fuss (perejil), *Pimpinella anisum* L. (anis), *Pinus caribaea* Morelet (pine), *Piper nigrum* L. (black pepper), *Plantago major* L. (llantén), *Syzygium aromaticum* L. (clove), *Thymus vulgaris* L. (thyme), *Verbena officinalis* L. (vervain), *Wedelia glauca* (Ortega) Hoffm (sunchillo). The selection of these plants was based in a bibliographic search, according to their antimicrobial properties[11,12].

Plant materials were dried in an oven stove at 60 ºC during 24 h and stored at room temperature until further processing. Dried plant material (aerial parts) was finely ground with a crushing machine. Ethanolic extracts of dried plants with pure ethanol (Merck 99 % of purity), and ethanol 70%-distilled water 30% were obtained. Ten g of this material was placed in 100 mL of respective solvents during 7 or 10 days at room temperature, keeping the extracts in the dark room and stirring them manually 3 times a day. The extracts were filtered by double gauze to eliminate the heaviest material and then filtered using Whatman filter paper Nº 1 (Whatman, England). The extracts were sterilized by filtration through 0.45 µm Millipore membrane filter. The essential oils (EO) were provided by the Food Industry Research Institute, Havana, Cuba. All were extracted by hydrodistillation using Clevenger type apparatus for 3 h.

Analysis of extracts by gas chromatography–mass spectrometry (GC–MS)

For GC/MS analysis a Perkin Elmer Clarus 500/560D equipped with a mass selective detector Elite-5MS fused silica column (25 m x 0.25 mm x 0.25 µm film thickness) were employed. The column temperature was programmed as follows: 80ºC hold 1 min, to 200ºC at 10ºC/min, then hold 10 min. Helium carrier gas was used at a flow rate of 0.8 mL/min, Split 10, Run time 23 min. Solvent Delay 0 a 3 min, MS Scan 50 a 400. The injector was maintained at 200ºC. Sample injection volume was 1 µL with a split ratio of 1:10. Mass spectra were recorded in the electron-impact (EI)

mode at 70 eV by 1.8 scans/s; the mass range used was m/z 35-400; ion source 180ºC and Intel line temperature were 250ºC.

Compounds were preliminarily identified by comparison of mass spectra with those of reference standards in NIST, as well as mass spectra from the literature.

Analysis of essential oils by gas chromatography–mass spectrometry (GC–MS)

For GC/MS analysis a HP 6890 Series II equipped with a mass selective detector HP-5973N and a HP-5MS fused silica column (25 m x 0.25 mm x 0.25 µm film thickness) were employed. The column temperature was programmed as follows: 70ºC hold 2 min, to 230ºC at 4ºC/min, and then hold 10 min. Helium carrier gas was used at a flow rate of 1 mL/min. The injector was maintained at 230ºC. Sample injection volume was 0.3 µL with a split ratio of 1:10. Mass spectra were recorded in the electron-impact (EI) mode at 70 eV by 1.8 scans/s; the mass range used was m/z 35-400; ion source and connecting parts temperature were 230ºC. Linear retention indices (RI) were calculated using n-paraffin standards.

Compounds were preliminarily identified by comparison of mass spectra with those of reference standards (FLAVORLIB library) or those in NIST, NBS/Wiley, as well as mass spectra from the literature, and then the identities of most were confirmed by comparison of their linear retention indices with those of reference standards or with published data[13].

Quantitative analysis was made by the normalization method from the electronic integration of the TIC peak areas without the use of correction factors.

Isolation and identification of microorganisms

The experiments were carried out with fungal and bacterial strains isolated from different documentary supports and indoor environments of repositories of the National Archive of Republic of Cuba (NARC) and Historical Archive of the Museum of La Plata (HAMLP), and Archive of Historical and Cartographic Research Department from the Geodesy Direction of Ministry of Public Works of Buenos Aires Province (AHCRD), both of Argentina[2,14]. The bacterial strains used were: *Bacillus polimixa, Bacillus cereus, Bacillus thuringiensis, Bacillus* sp.1, *Bacillus* sp.2, *Enterobacter agglomerans, Streptomyces* sp., and *Staphylococcus* sp. The fungal strains were *Aspergillus niger, Aspergillus clavatus, Penicillium* sp., *Fusarium* sp. They were maintained on malt extract agar (MEA) slants and the bacterial species had been maintained on nutrient agar (NA) slants.

Sampling procedures

Biofilms

Biofilms were removed from the surface of different materials, such as paper, silk maps and photographs deposited in the Archives, using a cotton swab. Samples were immediately placed into sterile plastic vessels[15] and taken to the laboratory. Swabs were homogenized in sterile physiologic solution and aliquots were seeded in adequate culture media[16]. Petri dishes were incubated at 28 ± 2 °C during 48 - 72 h for bacteria and one week for fungi.

Airborne microbiological sampling

The air samples were collected from two repositories using an impactation slot biocolector (slot II, 30 $L.min^{-1}$). The biocolector was placed at 1.5 m of height, following a diagonal sampling design. Petri dishes with NA were used for bacteria isolation and MEA for fungal growth.

Antimicrobial activity assay

The antimicrobial activity of the EO and extracts were evaluated by hole-plate diffusion methods[10,17]. Suspensions of the bacterial strains used were adjusted to tube 3 of the McFarland scale and the Petri dishes with NA were inoculated with a final concentration of 10^8 CFU/mL[7].

For the fungi, suspensions of conidia were adjusted using a Neubauer´s chamber to 10^6 conidia/mL[18] and the Petri dishes with MEA were inoculated with a final concentration of 10^4 conidia/mL[19].

Culture media (NA or MEA) were added in Petri dishes up to height of 4 mm. Six holes of 5 mm of diameter (d) were made equidistant, and 10 µL of each EO pure or dissolved in ethanol (70%) at different concentrations, and diverse extracts were added.

Ethanol at 70%, gentamycin sulphate at 40 mg/mL (Medical-pharmaceutical Industry, Cuba) and miconazole at 10 mg/mL (Medical-pharmaceutical Industry, Cuba) were used as controls. Each experiment

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was done in triplicate.

Petri dishes were incubated at 28 ± 2ºC for 24 h to 72 h for bacteria and during 5 days for fungal strains. The diameter of the inhibition zone was measured and it was not included of 5 mm of the holes. The established range to determine susceptibility to EO and extracts was evaluated according to the diameter (d) of inhibition zone: d ≤ 6 mm is indicative of negative activity; d = 6 – 9 mm indicates a moderate activity; d ≥ 9 mm indicates a positive activity[17].

RESULTS & DISCUSSION

The activity of the EO and plant extracts varied depending on the microorganism tested (TABLES 1, 2 and 3). In the literature has been indicated that the antimicrobial activity of the EO and plant extracts is due to different secondary metabolites present in them, including: triterpenoids, flavonoids, phenols, alkaloids, coumarins, tannins and steroids[20,21]. Some of them are present in the biocidal products evaluated (TABLE 4)

TABLE 1 : Activity of hydroalcoholic extracts from plants against biodeteriogen bacteria. Diameter of inhibition zone (mm) at 24h.

Plants		Microorganisms				
		Bacillus cereus	B.thuringiensis	Bacillus sp.1	Bacillus sp. 2	Staphylococcus sp.
Arctium lappa L.	70%		7	2/10*	0	
	99%		16	2/8*	12*	
Arctium sp.	70%	7	14		15	0
	99%	6	6		11	0
Artemisia sp.(leaves)	70%					20
Artemisia sp.(flowers)	70%					14
Baccharis crispa Pers	70%			3		
	99%			4		
Camellia sinensis (L.) Kuntze	70%			8	4	
Centaurea cyanus L	70%		0	0/5*	0	
	99%		8	2/8*	12*	
Cestrum nocturnum L.	99%	0	0	0		
Cichorium intybus L.	70%		0	10*		
	99%		6	0		
Conyza bonariensis (L.)Cronquist	70%	0	2		3	0
	99%	0	0		3	0
Cymbopogon citratus DC	70%			2		
	99%			3		
Eucalyptus citriodora	tincture		15	9		
Ilex paraguayensis	70%	0	2		4	0
	99%	0	2		2	0
Medicago sativa L.	70%		0	2		
	99%		0	0		
Nerium oleander L.	70%	4	5	10		
	99%	0	0	0		
Petroselinum crispum (Mill.)	70%			2		
	99%			0		
Pinnus caribaea Morelet	99%		13	6		
Plantago major L.	70%		0	0		
	99%		0	6*		
Verbena officinalis L.	99%	2	2	2		
Wedelia glauca (Ortega) Hoffm	70%					22

*** Bacteriostatic activity**

and the effect on the microbial strains tested is attributable to them. Some of the studied products produced spots on the papers (TABLE 5).

Positive activity with the hydroalcoholic extracts of *Arctium lappa* L. (99 %) *Arctium* sp. (70 and 99 %), *Eucalyptus citriodora* Hook, *Pinus caribaea* Morelet and *Wedelia glauca* (Ortega) Hoffm (70 %) was obtained with some of the bacterial strains analyzed (TABLE 1). The antimicrobial effect of *Arctium* sp. is attributed to a sesquiterpenic lactone present in the aerial parts of this plant[22]. Arctin and arctigenin, major constituents of the fruit of *Arctium lappa* L., exhibit various biological activities and a large number of pharmacological properties[23] *Eucalyptus citriodora* Hook and *Pinnus caribaea* Morelet are plants used in traditional medicine for their antibacterial and antifungal activity[24]. Antimicrobial activity of *Eucalyptus* sp. is due to the presence of tannins, terpenes and eucalyptol in their leaves. Studies using *Eucalyptus citriodora* Hook and *Pinnus caribaea* Morelet to prevent biodeterioration of papers exposed to artificial aging, showed no change in the appearance of them, as the acidity of the papers was unchanged[25].

For some bacterial strains hydroalcoholic extracts of *Arctium lappa* L., *Centaurea cyanus* L. *Cichorium intibus* L. and *Plantago major* L. presented bacteriostatic activity (TABLE 1).

EO had a positive activity in most of the strains tested (TABLE 2). The antibacterial activity of *Allium sativum* L. was highly variable among Gram-positive bacteria. Against *Bacillus thuringiensis* was moderate and positive against *Bacillus polymyxa.* This variability may be due to the resistance they have sporulated bacteria[26]. *Bacillus* spp. is a bacterium that can degrade a wide range of substrates given their physiological characteristics[27]. Furthermore, it has been reported that during the manufacturing process of the photographic paper, the genus *Bacillus* which can contaminate the gelatine that is part of emulsion[28]. Most species produce endospores that are highly resistant to extreme environmental conditions, antibiotics, disinfectants and other chemicals.

TABLE 2 : Activity of esential oils (EO) against biodeteriogen bacteria. Diameter of inhibition zone (mm) at 24 h.

Plants		EO	Microorganisms							
			B.cereus	B. thuringiensis	Bacillus sp. 1	Bacillus sp. 2	Staphylococcus sp.	Bacillus polymyxa	Enterobacter agglomerans	Streptomyces sp.
Allium sativum L.		pure	4	8	0			> 30	> 30	>30
		25%	0	7	10			11	> 30	>30
Arctium lappa L.		pure			7		8			
Citrus sinensis Osbeck		Pure	0	0				10	5	>30
		25%	0	3	10			4	2	8
Laurus nobilis L.		pure	6	8						
		25%	0	0	7			4	2	2
Mentha peperita L.		pure					4			
Mentha arvensis L.		pure					6			
Origanum vulgare L.		pure	18	17				11	15	17
		25%	>30	>30	10			10	10	11
Origanum x applii		pure					13			
Pimpinella anisum L.		pure	0	0	0			5	6	6
		25%	0	7	0			5	6	7
Piper auritum Kunth		5%		7	10*					
		10%		10	10*					
Piper nigrum L.		pure	2	2	0					
Syzygium aromaticum L.		pure	10	8	10	10				
		25%	18	24	7			10	6	12
Thymus vulgaris L.		pure	18	14	28	12				
		70%			40	14				
Controls	ethanol	70%	0	0	0	0	0	0	0	0
	Gentamycin sulphate	40mg/ mL	18	21	17			20	26	32

*** Bacteriostatic activity**

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Activity of garlic EO against *Enterobacter aglomerans* and *Streptomyces* sp. was positive. Action is likely to be due to allicin and ajoene[20], which are substances that inhibit the activity of sulphydric enzymes (choline esterase, urease, dehydrogenase triphosphate, i.a.) and not sulphydric (lactate dehydrogenase, alkaline phosphatase) of the microorganisms[29], but these compounds were not detected and it is possible that antifungal activity is due to the expense of the sulfides present in the EO. The antifungal activity was also strongly positive.

The antimicrobial activity of *Pimpinella anisum* L. EO was negative against *Bacillus* species tested (TABLE 2) and showed only moderate activity in the case of *Enterobacter agglomerans* and *Streptomyces* sp. The latter kind is considered since 1988 one of the most important with regard to occupational hazards[30].

Similar results were obtained by Kivanç and Akgül (1986)[31], and Chaudry and Tariq (2006)[32]. The principal compounds are anise and anethol anisaldehyde which possess amphiphilic properties that would interfere with the cytoplasmic membrane, proteins, lipids and other vital compounds for the bacterial cell.

The EO of *Pimpinella anisum* L. showed a positive and significant activity against all fungi tested (TABLE 3) similarly to that reported by Alpsoy (2010)[33].

For *Origanum vulgare* L. EO (TABLE 2), there were no significant differences between strains of Gram-positive and Gram-negative analyzed. It could be due to the presence of thymol, which can act on cell membranes[7]. However, for fungi was effective and inhibited also the formation of conidia of all strains tested (TABLE 3).

TABLE 3 : Activity of esential oils (EO) against biodeteriogen fungi. Diameter of inhibition zone (mm) at 5 days.

EO	Ethanol concentration	*Aspergillus niger*	*Aspergillus clavatus*	*Penicillium* sp.	*Fusarium* sp.
	50%	> 40	> 40	> 40	> 40
Allium sativum L.	25%	> 40	> 40	> 40	> 40
	7.5%	> 40	> 40	> 40	> 40
	50%	IS	IS	IS	IS
Citrus sinensis Osbeck	25%	IS	IS	IS	IS
	7.5%	IS	IS	IS	IS
	50%	20*	25*	30*	25*
Origanum vulgare L.	25%	15*	15*	15*	15*
	7.5%	5	8	9	9
	50%	> 40	> 40	> 40	> 40
Pimpinella anisum L.	25%	> 40	> 40	> 40	> 40
	7.5%	> 40	> 40	> 40	> 40
	50%	15*	13*	15*	20*
Syzygium aromaticum	25%	15*	7*	6*	15*
	7.5%	4	6	5	8

IS: inhibit sporulation; *: Indicates that also inhibit sporulation

The antibacterial and antifungal activity of *Syzygium aromaticum* L. EO was variable. Most Gram-positive bacteria were sensitive to this EO. With regard to the fungi, was able to inhibit both growth and sporulation at a concentration of 50% and had a significant effectiveness for *A. niger* and *Fusarium* sp. even at a concentration of 25%. Similar results were reported by other authors for documentary heritage conservation[9]. The antimicrobial activity of clove EO is attributed to eugenol (2-methoxy-4-allyl phenol). Clove EO contains high eugenol content (> 70%). It is an antimicrobial compound having wide spectra[34]. Laurel EO showed antibacterial and antifungal activity to low activity contrary to the report of Alpsoy (2010)[33]. *Aspergillus* spp. is one of the fungi of clinical interest, because it has species that are capable of causing a lot of damages to people, such as Type I allergies (immediate hypersensitivity or allergic rhinitis followed by asthma attacks), sinusitis, otitis, keratitis and can lead to severe aspergillosis[4,5].

The EO of *Thymus vulgaris* L. showed a positive activity against strains of *Bacillus* spp. tested.

TABLE 4 : Main compounds of EO and plant extracts by GC-MS.

EO	Main compounds
Allium sativum L.	Ajoene, di-2-propenyl disulfide, sulfoxide, di-2-propenyl trisulfide, methyl 2-propenyl trisulfide, di-2-propenyl sylph, methyl 2-propenyl disulfide, methyl 2-propenyl sylph,dimethyl trisulfide
Citrus sinensis Osbeck	Limonene, cineol, α-pinene, citral, citronellol
Laurus nobilis L.	Linalool, cineole, eugenol, sabinene, methyl eugenol, β-pinene, α- terpineol, β-caryophyllene
Mentha arvensis	Limonene, terpenes
Mentha piperita	Limonene, terpenes
Pimpinella anisum L.	Anethole, methyl chavicol, methyl eugenol, linalool, acetanisole
Piper nigrum L.	Terpenes
Origanum vulgare L.	*Trans*-β-cariofilen, 2-metil-5-1-metiletil fenol, Thymol, *cis*-β-terpineol, terpinen-4-ol, γ-terpinene,α-terpinene, *p*-cymene, sabine, and carvacrol
Origanum x applii	Thymol, carvacrol
Tymus vulgais L.	Terpenes, esters, carvacrol, terpenoids
Syzygium aromaticum L.	Eugenol, limonene, eugenyl acetate, methyl or methyl-hidroxibenzoate, anethole
Extracts	
Arctium lappa L.	citronellyl acetate, ketone, arctin
Artemisia sp.	Terpenes
Camellia sinensis (L.)Kuntze	Sulphurous, terpene
Centaurea cyanus L	I-docoseno, cetona, (-)-*trans*-pinano
Conyza bonariensis (L.)Cronquist	Terpenes
Nerium oleander L.	Terpenes
Verbena officinalis L.	Naphthalene, alkanes, *trans*-pinane, tricosane

TABLE 5 : Determination of stains on the paper after 6 months of applying drops of EO.

Product	Presence of stain	Color stain
Nerium oleander L.	+	yellow-greenish
Cestrum nocturnum L.	+	green [a]
Verbena officinalis L.	+	green [a]
Wedelia glauca(Ortega) Hoffm	+	green [a]
Citrus sinensis Osbeck	+	light yellow
Syzygium aromaticum L.	+	Yellow
Allium sativum L.	+/-	very light green
Origanum vulgare	+	Yellow
Thymus vulgaris L.	-	imperceptible even to the light
Pimpinella anisum L.	+	imperceptible even to the light
Nerium oleander L.	+/-	very light yellow
Piper nigrum L.	+/-	very light yellow

[a]: Indicates that the chlorophyll of the extract stain the paper

CONCLUSIONS

Biocidal effect was observed in many of the extracts and EO tested against strains of bacteria and fungi isolated from air and documents belonging to the Argentine and Cuban documentary heritage.

The results obtained it follows the promising use of these environmentally friendly products from plants in the control of microorganisms associated with the biodeterioration of cultural heritage.

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