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Environmentally friendly extracts from *Eucalyptus citriodora* Hook. and *Pinus caribaea* Morelet their application in the control of the biofilms in biodeterioration on paper

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ABSTRACT

Biodeterioration of archival materials involves alteration of physicochemical and mechanical properties by organisms. This causes loss of aesthetic properties and irreversible degradation of documents. Since ancient times, extracts of plants have been used as antimicrobials in various fields. The use of such environmentally friendly products in the biodeterioration field is viable and has economic, environmental and ecological advantages. The aims of this research were: i) qualitative identification of secondary metabolites in natural extracts of *Eucalyptus citriodora* Hook. and *Pinus caribaea* Morelet, ii) evaluation of the biocidal activity of the extracts on adherence to paper and biofilm formation by *Bacillus* sp., iii) evaluation of the antimicrobial effects of the extracts on *Bacillus* sp. and *Bacillus thuringiensis* isolated from archival materials. Compounds identified in the extracts included alkaloids, cumarines, flavonoids, phenols, tannins, terpenes and steroids. The antimicrobial activity was studied by the agar diffusion technique. Significant holes of inhibition were obtained. Aged papers adsorbed with extracts in the presence of *Bacillus* sp were tested. A decrease of microorganisms adhesion and biofilm formation to paper adsorbed with extracts was observed.

KEYWORDS

Archival materials;
Biodeterioration;
Microorganisms;
Natural extracts.

INTRODUCTION

Historical documents stored in archives or museums are permanently subject to physical, chemical, and/or biological alterations. Biological deterioration is caused by microorganisms through their adherence and biofilm formation and use of the paper as a source of carbon and energy. Biodeterioration causes unwanted changes to the material properties. Various microbial populations, bacteria, fungi and actinomycetes, interact in adherence and paper degradation. Paper, which is composed of vegetable fibers, functional additives (glue,

filling, optical polishing and consolidating agents) and ink with organic binders, is vulnerable to abiotic and biotic factors. The damage of documentary material can result in an ecological succession of microorganisms, biofilms or consortia that potentiate the deterioration of the documents[5,19,24]. Fungi, like many bacteria, produce spots of different colours on materials, which have been associated with a process called foxing[3,11,43]. Foxing is the result of the release and excretion of pigments and acids by microorganisms, causing the appearance of brown yellow spots. While their chemical or biological origin is discussed, damage can be initiated by microorganisms on paper. The action of microorganisms in paper biodeterioration results in changes of chemical composition of fibrous and non fibrous materials; the efficient multi-enzyme systems of microorganisms enable them to use different nutrient sources under varied environmental conditions. *Bacillus* sp., for example, may participate in the deterioration of paper by the action of cellulase and xylanase enzymes that hydrolyze the starch used as sizing, producing acids that eventually accelerate the deterioration of the cellulose fiber[14,30] and metabolites such as lactic acid, causing pH decrease and leading to violaceous or reddish stains and destruction of the paper[5,19,25,39,41].

Bacillus and *Streptomyces* genus has cellulolytic and lignolytic activity and are able to excrete hydrolytic enzymes such as proteases and chitinases[1] that can degrade the proteins and chitin in the fungal wall[29].

Chemicals for routine prevention of paper biodeterioration and in response to infestations have been traditionally used. However these are not always effective and certainly do not correct the damage already caused. Currently, chemicals are decreasingly used because of the risk that these represent to health of the staff, to the material and to the environment. Therefore, the choice of a product for control and prevention of biodeterioration becomes increasingly more restricted due to stringent requirements for their use[6,9,15,16,17]. In the past few years, there has been increasing interest in silver nanoparticles and in the use of natural substances because of their high antibacterial and antifungal properties[17,21,34]. It is generally considered that compound produced naturally, rather than synthetically, will be biodegraded more easily and will therefore be more environmentally acceptable. This assumption is based on the known diversity and adaptability of microorganisms which can degrade most of the substances naturally occurring on this planet[15].

However, among several studies reported, only a few mentions their actual use in the field of conservation of cultural properties. Antimicrobial agents isolated from plants can control intermediary metabolism, activating or inhibiting enzymatic reactions, directly affecting an enzymatic synthesis, or changing membrane structures[35,38]. The major part of the compounds with antimicrobial activity found in plants, herbs and dried species are phenolics, terpenes, aliphatics, aldehydes, ketones, acids and isoflavonoids[22]. The aims of this research were: i) qualitative identification of secondary metabolites in natural extracts of *Eucalyptus citriodora* Hook. and *Pinus caribaea* Morelet, ii) evaluation of the biocidal activity of the extracts on adherence to paper and biofilm formation by *Bacillus* sp., iii) evaluation of the antimicrobial effects of the extracts on *Bacillus* sp. and *Bacillus thuringiensis* isolated from archival materials.

EXPERIMENTAL

Vegetable material, extraction and phytochemical analysis

Leaves of adults *Eucalyptus citriodora* Hook. (*E. citriodora* Hook.) and *Pinus caribaea* Morelet (*P. caribaea* Morelet) were collected in the natural habitat in the province of Havana, Republic of Cuba. Plants were in a vegetative state. Leaves were washed and drained in an oven with air recirculation for 7 days and then pulverized in a blade mill. After taxonomic classification, samples were deposited in the herbarium of the National Archives (0002 ARNAC *Eucalyptus* and ARNAC 003 *Pinus*). Extract of *E. citriodora* Hook. and tincture of *P. caribaea* Morelet were obtained by repercolation in 70% ethanol and sterilized by 0.22 µm Millipore membrane filtration (drug / extract relationship is usually 1:1 or 1:2 and 1:5 o 1:10 respectively).

The physicochemical characteristics of the extracts were determined following the methodology accepted by the Ministry of Public Health of the Republic of Cuba[27,28], and the identification of secondary metabo-

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lites following Sanabria-Gallindo et al.1997[33] All determinations were performed in triplicate.

Sampling, isolation and identification of microorganisms.

Samples were taken from the surfaces of photographic paper with fox-like reddish-brown colour spots, type "foxing" (Figure 1) in the Historical Archive of the Museum of La Plata (HAMLP), Argentina, with sterile cotton swabs. They were homogenized in 10 ml of sterile saline and inoculated on Plate Count Agar (PCA media) for to grow heterotrophic mesophilic bacteria[17,19,26]. Bacteria were typified according to the Gram stain and biochemical tests[36]. *Bacillus* spp. were identified by molecular techniques (16S rRNA gene), with NCBI accession number EU184084 = *Bacillus* sp. and NCBI accession number AM747224= *Bacillus thuringiensis*[19,20].

Figure 1 : Foxing spots in a phothograph from HAMLP

***"In vitro"* test for antimicrobial activity**

Bacillus sp. and *Bacillus thuringiensis* were grown in nutrient agar for 24 h. The antimicrobial activity of *E. citriodora* Hook. and *P. caribaea* Morelet was determined by the hole technique[40]. The inoculum used for this technique corresponded to the 3rd tube of the McFarland scale (1x 10^6 CFU/ml)[31], Ten µl of the extract and tincture at 5 and 10% were added to holes of 5 mm diameter. Controls were sterile distilled water and 70% ethanol in equal volume. The holes were equidistant (6 peripheral wells/plate). Well diameter was not taken into account when measuring the inhibition halo. After incubating the dishes for 24 h at 28°C, holes were measured. Tests were performed in triplicate.

Laboratory assays: bioadhesion and biofilm formation of *Bacillus* sp. to paper

From a pure culture of *Bacillus* sp. (24 h incubation), an aliquot was taken and diluted in saline to reach turbidity of tube 3 on the McFarland scale. For growth of *Bacillus* sp. 1 ml of this suspension was seeded on solid mineral medium whose composition was sodium nitrate 2 g; dipotassium phosphate 1 g. magnesium sulphate 0.5 g; potassium chloride 0.5 g; yeast extract 0.5 g; ferrous sulphate 0.01 g; agar 20 g, per 1 l; pH = 5.5 and Petri dished were incubated 24 h.

A strip of aged sterilized filter paper of 2 cm^2 (Papel Archive Text. Ref. 678-70A4) (72 h at 105 °C, corresponding to 25 years of aging) adsorbed with *E. citriodora* Hook. and *P. caribaea* Morelet (Figure 2) were placed on the Petri dished. Aged papers without adsorbed extracts were used as controls. Strain that showed better growth and bioadhesion using the paper as sole carbon source was selected for studies.

Accelerated aging method used was described by Browning (1969)[7] based on the equation Arrehenius:

$$k = s(-Ea / RT)$$

where: k = constant of the reaction; Ea = Energy of activation R = constant of gas; T = Temperature absolute of process in Kelvin; S = factor of frecuency

Two papers were removed every 5, 24 and 48 h; one of them was used for evaluating adhered cells by plating and biofilm formation; and the other for Scanning Electron Microscopy (SEM). After incubation, papers were washed with sterilized distilled water to remove surplus agar and unattached bacteria. For microbial counting the paper were submerged in sterile saline, shaken slightly and then sonicated. Decimal dilutions were spread on nutrient agar, incubated at 28 ºC for 24 h and counting CFU/cm^2. The tests were performed in quadruplet.

Monitoring of bioadhesion and biofilm formation by SEM

Bioadhesion and biofilm formation to papers were observed by SEM Jeol 6360 LV. Papers were dried at room temperature and then were kept in a closed chamber with pure ethanol for 24 h and metalized with Au/Pd previous to observation.

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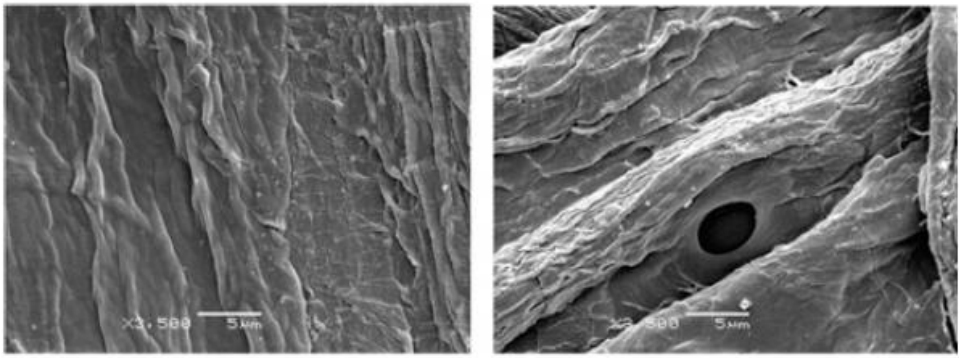

Figure 2 : SEM image of paper adsorbed with A) *Eucalyptus citriodora* Hook., and B) *Pinus caribaea* Morelet (3500X) Bar = 5µm.

TABLE 1 : Results of secondary metabolites identified of extract and tincture

ASSAYS	Secundary metabolites	*E. citriodora* Hook.	*P. caribaea* Morelet
Dragendorff	Alcaloids	+	+
Mayer	Alcaloids	+	+
Wagner	Alcaloids	+	+
Liebermann Burchard	Triterpenes, steroids	+	++
Sudán III	Oils, fats	++	+
Fehling	Reducing sugars	+	+
Cloruros	Phenols, taninns	+++	+++
Shinoda	Flavonoids	+++	+++

(+, ++, +++) Increasing amount

TABLE 2 : Physicochemical characteristics of extract and tincture

	Total solids (mg/dL)	Refractive index	pH	Relative density	Alcoholic content (%)
E. citriodora Hook.	6.00	1.375	4.75	0.90	65.0
P. caribaea Morelet	6.50	1.325	5.34	0.94	51.5

RESULTS AND DISCUSSION

The results of qualitative assays for secondary metabolites are shown in TABLE 1. Flavonoids and phenols were abundant in both samples, while the other metabolites showed variability. In the case of phenols, individual trials were characterized by intense colors, which show the wide variety of hydroxylated structures.

There was no qualitative similarity between samples with respect to steroid and triterpenoid contents. Steroids were detected most abundantly in the tincture and the test applied was characterized by an intense blue-green color, which shows the presence of several sterols in the tissue of the plant[18].

The mean values of physicochemical determinations of the extracts are shown in TABLE 2.

Of the metabolites detected in both species, four of them have proven antibacterial activity: triterpenes, flavonoids, phenols and tannins[4].

The values for mean and standard deviation from the growth inhibition holes (mm) for the extracts were: *Bacillus* sp. with *E. citriodora* Hook. 12 ± 0.3 and *P. caribea* Morelet 10 ± 0.3; *Bacillus thuringiensis* with *E. citriodora* Hook. 15 ± 0.4 and *P. caribea* Morelet 13 ± 0.3. Values lower than 6 mm are negative, moderate between 6 and 9 mm and positive more than 9 mm. Control assayed with ethanol and sterile water were negative.

As in previous investigations[18], positive results were given with this isolate. The importance of this research is that these spore forming microorganisms showed in-

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TABLE 3 : Adherent *Bacillus* sp. on papers adsorbated with extracts after 5, 24 and 48 hs. expressed in UFC/cm²

	Eucalyptus citriodora Hook.			*Pinus caribaea* Morelet			Control		
	5 h	24 h	48 h	5 h	24 h	48 h	5 h	24 h	48 h
X	37.33	75.67	170	6.33	8.67	25	60.33	186.33	220
SD	8.74	10.79	8	3.51	3.51	5	9.61	7.21	7.21

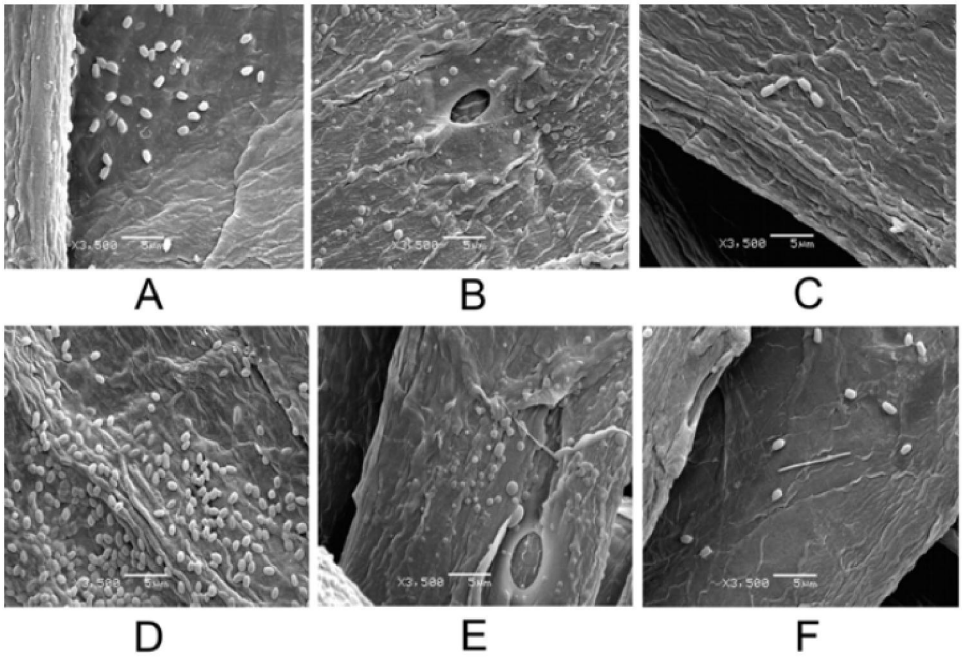

Figure 3 : SEM images. Aged control paper in *Bacillus* sp. culture after 24 h A) and 48 h D); aged paper adsorbed with *Eucalyptus citriodora* Hook. in *Bacillus* sp. culture after. 24 h B) and 48 h E); aged paper adsorbed with *Pinus caribaea* Morelet in *Bacillus* sp. culture after 24 h C) and 48 h F). (3500X) Bar = 5µm.

hibition holes.

Phytochemical studies on leaves and bark of the Pinaceae family have shown the presence of triterpene acids, which may contribute to the antimicrobial activity, their mechanism of action involving the disruption of the microbial membrane[42]. *E. citriodora* Hook. and *P. caribaea* Morelet plants have been widely studied, mainly due to their use in traditional medicine and their antibacterial and antifungal activity. Antimicrobial activity is attributed to the presence of tannins, terpenes and eucalyptol in leaves of the genus *Eucalyptus*[32]. Some of these are present in the solutions evaluated here and they could be responsible for the antimicrobial effect noted (TABLE 1).

Bacillus sp. showed the highest bioadhesion counts on the control paper (TABLE 3). On papers absorbed with extract the number of UFC/cm² in biofilm formation decreased, confirming the effect of the extracts (TABLE 3). *P. caribaea* Morelet was most effective in the inhibition of adherence of *Bacillus* sp to paper that *E citriodora* Hook. (TABLE 3).

The biofilm formation on control paper and the decreased of *Bacillus* sp. adherence on aged papers adsorbed with extracts of *E. citriodora* Hook., and *P. caribaea* Morelet were corroborated by SEM (Figure 3).

Bacillus sp. may participate in the biodeterioration of paper through cellulase and xylanase action on starch, which is used as sizing in paper manufacturing; acid production by the bacterial cells will also, over time, accelerate the deterioration of cellulose fibers[14,24]. During the manufacturing process of the photographic paper, *Bacillus* spp. can degrade the gelatin of the emulsion[8,37]. The lack of control of microorganisms can significantly detract from the economic value of the enormous range of products[13,23]. It has been estimated that losses caused by biodeterioration are about 1% (billions of dollars)[2].

Acidity is one of the more important causes of paper deterioration; there are standards based almost exclusively on determination of acidity to predict perma-

nence. The determination of pH is one of the more reliable methods to measure acidity, which may be used on paper because of the conductivity generated in the presence of humidity, de la Paz et al. (2009)[10] have demonstrated that these natural extracts not favor the acid formation on the paper as not only the pH remained invariable.

CONCLUSIONS

- A decrease of *Bacillus* sp. bioadhesion and biofilm formation to paper adsorbed with extracts was observed.
- Results obtained in this research corroborate the antimicrobial activity of environmentally friendly extracts of *Eucalyptus citriodora* Hook. and tincture of *Pinus caribaea* Morelet.
- Alcaloids, triterpenes, steroids, phenols, present in the solutions evaluated could be responsible for the antimicrobial effect noted.
- This would support their promising use to control microorganisms associated with biodeterioration of archival materials, allowing the preservation of the documentary heritage.

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