



BIOCIDAL ACTIVITY OF TWO ESSENTIAL OILS ON FUNGI THAT CAUSE DEGRADATION OF PAPER DOCUMENTS

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Abstract

*The aim of this study was to evaluate the biocidal activity of essential oils of *Syzygium aromaticum* (L.) Merr. and L. M. Perry (nail) and *Allium sativum* L. (garlic) against different fungal species producing paper degradation and deterioration. Essential oils (EOs) were obtained from harvested plants in their natural habitat in Cuba, and were tested against the species *Aspergillus niger*, *Aspergillus flavus* and *Penicillium* sp. which were isolated from archival indoor environments and documents with patrimonial value. The biocidal activity was studied at different concentrations (70, 50, 25, 12.5 and 7.5%) using the agar diffusion method. The effect of extracts on paper alterations was studied through different techniques including determination of pH and number of copper and scanning electron microscopy (SEM) observations. EOs were analyzed by gas chromatography coupled to mass spectroscopy (GC/MS). The determination of inhibition zones by the agar diffusion method of the tested EOs showed a moderate and/or positive effect. The study of the antifungal activity on paper ("in vivo") shows that both clove and garlic oils were potent biocides. Although the paper structure was not affected by EOs pure, some molecular damages were observed at lower concentrations across determinations of the pH and copper number.*

Keywords: Degradation; Documents; Essential oils; Fungi; Paper; SEM.

Introduction

A wide range of materials, such as metals, paints, paper, paperboard, rocks, photos, textiles, leather, plastics, etc. can be affected by microclimatic conditions (temperature and relative humidity) and can suffer from physical, chemical and aesthetics damage caused by insects, algae, lichen, fungi and bacteria [1, 2]. Degradation by fungi of artworks and documents on paper is a particularly complex phenomenon because it involves living microorganisms interacting with highly heterogeneous materials, such as paper and others. Biodegradation and biofouling are due to microbiological, biological and physicochemical processes [3].

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The biodegradation of organic material is an essential environmental process that recycles complex organic matter and is an integral component of life. However, this process also destroys historical documents composed mainly of organic matter, especially natural and synthetic polymers, resulting in a loss of valuable cultural properties stored in libraries, archives and museums [4].

The action of microorganisms during the paper biodegradation process causes changes in the 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. Fungi and many bacteria produce spots of different colors on materials, which have been associated with a process called foxing [5, 6].

It has been estimated that at least 99 % of the world's microbial biomass exists in the form of biofilms, a complex differentiated to surface-associated community embedded in a self-produced polymeric matrix enabling to the microorganisms to develop coordinated and efficient survival strategies. Although the inclination to colonize surfaces is advantageous from the microbial standpoint, this may cause chronic infections [7], parasitism phenomena in animals and plants [8], deterioration of historical and artistic objects [2, 9], biodegradation of engineered systems [10], and fouling in food-processing equipments [11]. Furthermore, the injury caused by the biofilm has a profound socio-economic impact, incurring costs that result in a huge financial burden for the institutions.

Chemicals for routine prevention of paper degradation and deterioration by microorganisms and in response to infestations have been traditionally used. However, they are not always effective and do not correct the damage already caused. Chemicals that have traditionally been used as antimicrobials to prevent deterioration by microorganisms of cultural heritage generally pollute the environment, affect the human health and cause severe changes in the material on which they are applied.

The best way to avoid and eradicate the fungal infestation which cause degradation of different materials is to use antifungal compounds of botanical origin that are generally considered safe for human health and the environment [12-15]. These biocides are used in plant protection, food processing and the cosmetic industry as well as in the production of packaging, medical, finishing and insulation materials containing natural fibers. They are also used to preserve museum exhibits and antique book collections, important documents and different materials [16, 17].

The plant essential oils are a mixture of different components and their biological activity is generally determined by their major components or synergism/antagonisms among different components [18]. Few papers address the inhibition of biofilm formation by using these compounds [19]. The aims of this paper were: i) to assess the biocidal activity of essential oils (EOs) of *Syzygium aromaticum* (L.) Merr. & L. M. Perry (clove) and *Allium sativum* L. (garlic) against different fungal species isolated from archival indoor environment and damaged documents with heritage value and ii) to study the effect of EOs on alterations of papers.

Materials and methods

Obtaining of essential oils from plants

The plants *Allium sativum* L. (garlic) and *Syzygium aromaticum* (L.) Merr. & L. M. Perry (clove) were collected from their natural habitat in Cuba and the essential oils (EOs) were provided by the Food Industry Research Institute, Havana, Cuba. The oils were obtained by hydrodistillation using a Clevenger type apparatus for 3 hours.

Fungal strains used

Three fungal strains were used in the research. *Aspergillus flavus* and *Penicillium* sp. were isolated from indoor environments of the repositories of the National Archive of the

Republic of Cuba (NARC) using an impactation slot biocolector (slot II, 30L·min⁻¹), which was placed at 1.5m height [1] and *Aspergillus niger* was isolated from a paper map by removing the biofilm from the surface using a cotton swab [20]. In both cases the culture media Malt Extract Agar (MEA) with 7.5% NaCl was used to isolate the fungal strains [21].

Analysis of EOs by gas chromatography mass spectrometry (GC-MS).

For GC/MS analysis, an HP 6890 Series II equipped with a mass-selective detector HP-5973N and an HP-5MS-fused silica column (25m × 0.25mm × 0.25µm film thickness) were employed. The column temperature was programmed as follows: 70°C holds 2 min to 230°C at 4 °C/min and then holds 10 min. Helium carrier gas was used at a flow rate of 1mL/min. The injector was maintained at 230°C. Sample injection volume was 0.3µL with a split ratio of 1:10. Mass spectra were recorded in the electron-impact (EI) mode at 70eV by 1.8 scans/s; the mass range used was m/z 35–400; ion source and connecting parts temperature was 230°C. Linear retention indices (RIs) 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, and mass spectra from the literature, and then the identities of most compounds were confirmed by comparison of their linear retention indices with those of reference standards or with published data.

Quantitative analysis was made by the normalization method from the electronic integration of the TIC peak areas without the use of correction factors [22].

Laboratory tests with fungal strains.

a. Test for antifungal activity "in vitro".

The antimicrobial activity of the EOs at different concentrations was evaluated by hole-plate diffusion methods. To this end, fungi suspensions of conidia of each strain were adjusted using a Neubauer's chamber to 10⁶ conidia/mL [23] and the Petri dishes with MEA were inoculated with a final concentration of 10⁴ conidia/mL.

Culture media (MEA) were added in Petri dishes up to 4 mm height. Six holes of 5 mm of diameter were made equidistant and 10 µL of each EO dissolved in ethanol (70 %) at different concentrations (70, 50, 25, 12.5 and 7.5%) were added. Ethanol at 70 % and miconazole at 10 mg/mL (Medical-pharmaceutical Industry, Cuba) were used as controls. Each experiment was done in triplicate.

Petri dishes were incubated at 30±2°C for 5 days. Having finished the time of incubation, the diameter of the inhibition zone was measured and it was not included in 5mm of the holes. The established range to determine susceptibility to EO was evaluated according to the diameter (d) of inhibition zone: d ≤ 6 mm is indicative of negative activity; d = 7 - 10 mm indicates a moderate activity; d ≥ 11 mm indicates a positive activity [22].

b. Test for antifungal activity "in vivo" on paper.

Sterile paper strips (60 × 30mm) for laser impression quality were impregnated with each fungal suspension and were incubated in humidity chamber at 75 – 80% [24] at 30°C during 30 days. Each strip was treated with each EO at 37% and 12.5% dissolved in ethanol at 70% at 0, 5, 10, 15, 20, 25 and 30min. The strips were then washed with distilled water, seeded in MEA and incubated at 30°C for 7 days. Each experimental variant was done in triplicate.

c. Test for bioadhesion on paper.

Other sterile paper strips (60×30mm) for laser impression quality were impregnated with each EO at different concentration by separated. The concentrations of EO analyzed in this assay were 12.5 and 7.5% dissolved in ethanol at 70%. The paper strips without EO were used as a control. The strips dried were inoculated separately by each of three fungal strains conidia

suspension. The incubation was made in humidity chamber at 75 – 80% [24] at 30°C for 30 days. Having finished the time of incubation, fungal growth was observed by stereomicroscopic and optic microscopic.

Determination of damage produced in papers by EOs.

Leaves of paper at the same quality mentioned above were painted using a paintbrush with 12.5 and 6.25% of each EO dissolved with ethanol (70%). The leaves dried were artificially aged at 105°C for 72 h corresponding to 25 years of ageing (accelerated ageing) [25, 26]. Each experiment was done in triplicate.

a. Observation of the fibers by Scanning Electron Microscope (SEM).

Samples of paper with and without biocides (control) and with accelerated ageing or not were observed by scanning electron microscopy (SEM). In this case the essential oils were used in pure form. The samples were monitored and observed by SEM (Jeol 6360 LV). They were then kept in a closed chamber with pure ethanol for 24h and metalized with Au/Pd prior to observation.

b. Determination of the pH.

Paper pH knowledge is a simple but a very useful tool to define the paper conservation status. The method used determines the pH value of a thin water layer laid on a surface by a potentiometric measurement. The water allows the substances present in the superficial paper layer to cross into the water solution. These substances affect pH values.

The pH value was determined by using a portable pH-meter (Digi-Sense, USA) connected to a plane electrode of contact. The measurements were carried out in five areas of each leave adding a drop of water and placing the sensor previously there above [27].

c. Determination of the copper number.

Other leaves with same EO treatments were cut in small fragments and 1.5g of these papers were put in a hot bath at 100 °C with a mixture of 5mL of CuSO₄ and 95mL of Na₂CO₃ – NaHCO₃ d for 3h. The fragments were filtered and washed with 100mL of Na₂CO₃ at 20°C, then with 250mL of distilled water at 95°C. The filtered were discarded and the paper fragments were treated with 25mL of molybdophosphoric acid. The fragments were then filtered and washed with cold distilled water until 700mL of the final volume. This solution was titrated with potassium permanganate solution at 0.05N [28]. The determination was done in triplicate in each case.

$$\text{Copper number} = 6.36 \times V \times N/W$$

Where V: volume of potassium permanganate solution consumed in the titration, N: normality of the potassium permanganate solution, W: weight of the sample.

Statistical analysis

The ANOVA-1 and multiple range tests were used to evaluate the damage produced on papers by the EOs in relation to the copper number and pH of paper and accordingly to the differences between the papers treated with each EO and paper control. The assessment was conducted using the STATGRAPHICS Plus-5 program. Results with $P \leq 0.05$ were considered statistically significant.

Results and discussion

The results show that in the clove oil a phenolic compound (eugenol) was the main component detected (Table 1). This result is consistent with previous reports [29]. The other

compounds identified have been isolated from clove oil by other authors too [16, 22]. In garlic oil the sulphured components were the predominant ones.

Table 1. The essential oils tested and the major components detected in each one by gas chromatography–mass spectrometry (GC–MS).

Common name	Botanical correspondence	Percentage composition of major components
Clove	<i>Syzygium aromaticum</i> (L.) Merr. & L. M. Perry	Eugenol (67.0%), eugenylacetate (18.1%), methyl-hydroxybenzoate (9.0%), anethole (5.9%)
Garlic	<i>Allium sativum</i> L.	Di-2-propenyl trisulphide (31.9%), methyl 2-propenyltrisulphide (21.7%), di-2-propenyl disulphide (20.7%), di-2-propenylsulphide (7.9%), methyl 2-propenylsulphide (5.6%), dimethyl trisulphide (1.6%)

The inhibition zones obtained for each fungus showed that the EOs exhibited different antifungal effects (Table 2). The garlic oil showed the highest activity while the clove oil showed a considerable variation in the inhibition zones, sizes ranging from 20 – 5mm among fungal isolates, depending on their sensitivity and the concentrations. This EO was efficient until 25% on the inhibition zones with diameters higher than 11 mm. Antifungal activity of clove oil at 12.5% was high only against *Aspergillus niger*, while against *Aspergillus clavatus* and *Penicillium* sp. was lower. Furthermore, it was observed that the clove oil stopped the fungal sporulation in the area next to the lack of growth in all concentrations tested (Table 2 and Fig. 1). In this area the conidiogenesis was totally inhibited (Fig. 2). Similar results were reported previously by other authors [30, 31].

Table 2. Median values of the antifungal activity of five essential oils (EOs) concentrations dissolved in ethanol at 70% (v/v) against three fungal strains isolated from documents biofilms and indoor environments of archival repositories.

Diameter of inhibition zone (mm) at 5 days ^a				
EOs	Conc. (%)	<i>Aspergillus niger</i>	<i>Aspergillus flavus</i>	<i>Penicillium</i> sp.
Clove	70.0	20 *	15 *	20 *
	50.0	15 *	13 *	15 *
	25.0	13 *	12 *	13 *
	12.5	11 *	7 *	6 *
	7.5	8 *	6 *	5 *
Garlic	70.0	40	40	40
	50.0	40	40	40
	25.0	40	40	40
	12.5	40	40	40
	7.5	40	40	40
Ethanol	70	0	0	0
Miconazole	10 mg/mL	6	6	10

^a: Data are mean of three replications;

*: It indicates that the oil used stops fungal sporulation in the area near the lack of growth.

The miconazole used as positive control (10 mg/mL) was less effective than the oils; and no inhibition zones were observed within the negative control (ethanol at 70%). It is known that phenolic compounds as eugenol are strong inhibitors of the enzymatic processes in the microbial membranes due the lipophilic characteristics of the free OH groups.

This inhibition affects the vital processes of the microbial cell as osmosis and biosynthesis of sterols and phospholipids [32].

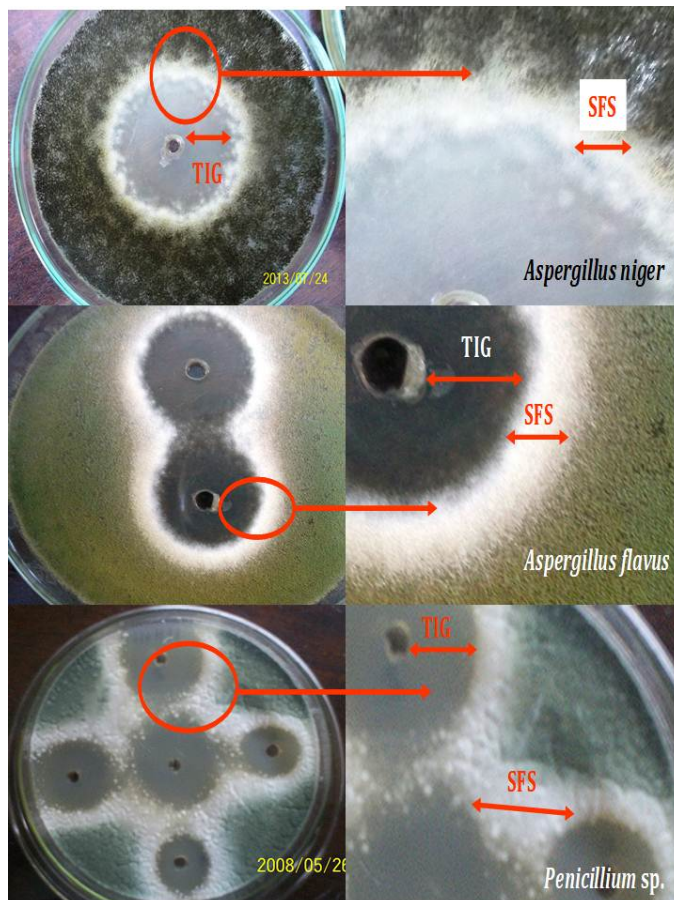


Fig. 1. Behavior of the antifungal activity of the clove oil dissolved in ethanol (70%) against the fungal strains isolated from documents and the archival indoor environments, along with the corresponding clove oil concentration.

TIG: Indicate Total Inhibition of Growth; SFS: Indicate Stop of Fungal Sporulation.

Recently, the action mechanism of eugenol and anethole on yeasts and filamentous fungi was exposed. According to a previous report [29], an extensive lesion of the cell membrane and considerable reduction in the quantity of ergosterol were caused by clove oil and eugenol. Furthermore, Rana et al [33] reported a profound effect and clearly visible in the form (deformities/distortion) and structure of the spores in the presence of clove oil and eugenol. Also, some authors have suggested that eugenol could apply as supplementary antifungal agents [34].

It is important to highlight the fact that the fungicidal activity of clove oil was lower than that of the garlic oil. However, clove oil is used as biocide, antiseptic [33] and the volatile vapors have been reported as antifungal [35].

In relation to garlic oil the growth was completely inhibited. However, the ajoene or ajoene-derived were not found; and these substances have a high antifungal activity [36]. These compounds inhibit the activity of sulphhydryl enzymes (choline esterase, urease, dehydrogenase

triphosphate, i.e.) and non sulphhydic enzymes (lactate dehydrogenase, alkaline phosphatase) of the microorganisms [37].

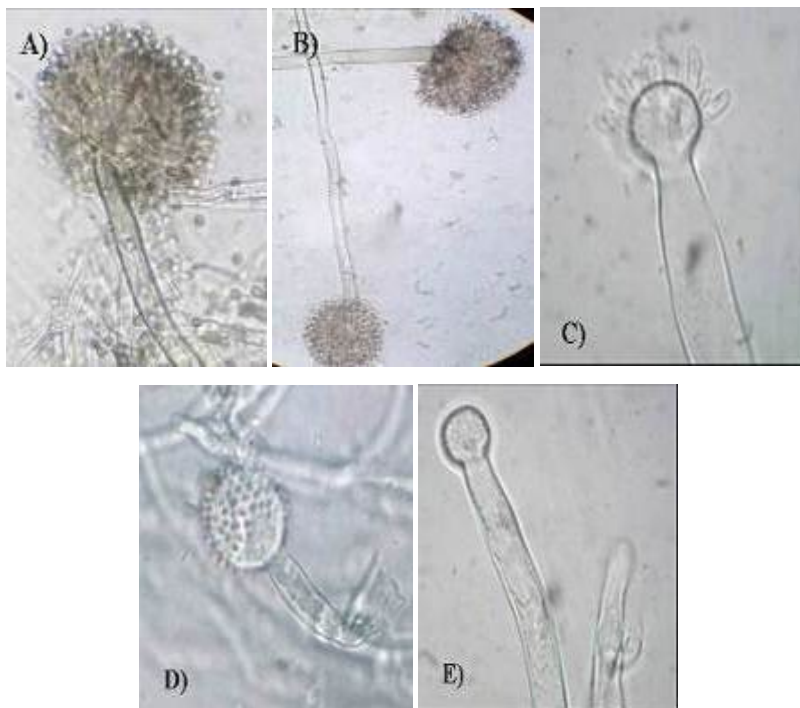


Fig. 2. Conidiophore appearance of *Aspergillus niger* in the absence and presence of the clove oil: A and B - microscopic images of conidiophores of this fungal specie in the control sample (100 X) (no EO); C, D and E - microscopic images showing the absence of conidia (100 X), these images were taken from SFS area shown in fig. 1.

Nevertheless, in the garlic oil a high quantity of sulfur-containing compounds was detected, which confers a potent biocidal activity at all tested concentrations. With this EO, the activity was very high in all cases. The volatilization of EO vapors could contribute to the increase of this activity [38] although the micro atmosphere formed inside a Petri dish does not reflect a real situation; it is a useful method for preliminary studies. On the other hand, it is the methodology reported for studies “*in vitro*” of antimicrobial activity.

When the antifungal activity on paper (“*in vivo*”) was evaluated, a total biocide activity was observed for the two EOs at different concentrations and times. The growth of the three fungal strains was inhibited immediately after being in contact with any one of the concentrations of two essentials oils analyzed (0 min of contact). Is important to highlight that the highest killer effect of the EOs on the three fungal species was obtained at the lowest concentration studied (7.5%); this result is contrary to that obtained in presence of culture media (“*in vitro*”), possibly because the diffusion effect of EOs in the agar medium incite a lower antifungal activity. However, a strong biocidal effect was proved by these two EOs both “*in vitro*” and “*in vivo*” tests and these results suggest the necessity to continue the studies, including further studies of the two Eos at lower concentrations.

When the paper was previously treated with the oils and incubated in the humidity chamber, the fungal growth didn’t developed and the bioadhesion of the three fungal species was inhibited by these EOs at the lowest concentration studied (7.5 %). Although these results

are preliminary, it was observed that the three fungal strains were unable to form biofilms. Nevertheless, more studies are necessary to confirm this preliminary result.

Although few papers address the inhibition of biofilms formation by using natural compounds from plants, those studies have focused on the inhibition of bacterial biofilms principally [39] and in levaduriform fungi in particular *Candida albicans* [40]. However, recently it was reported the inhibition of microbial biofilm formation by *Fusarium oxysporum* using clove EO and thyme [41].

Regarding the effect of the pure EOs of clove and garlic on the papers, the observations of SEM showed that the applications of the two biocides did not modify the structure of the paper before and after the artificial ageing. The paper fibres were not destroyed and apparently the structure, texture and length were maintained (Fig. 3). Nevertheless, the pH and the copper number showed different behaviors because some affectation was observed on the molecular characteristics of the paper.

Table 3 shows the results of the pH and the copper number determinations. Before accelerated ageing, most of the papers treated with EOs showed a marked decrease in the pH which is significantly different to that of the control. Similar results were obtained by Rakotonirainy et al. (2007) [38]. On the contrary, after accelerated ageing, the pH values obtained were lower in most of the papers treated with EO, while the pH was higher after the artificial ageing only with the clove oil at 7.5%. This effect was marked with garlic oil. It is normal that paper pH decreases during the process of natural ageing [26].

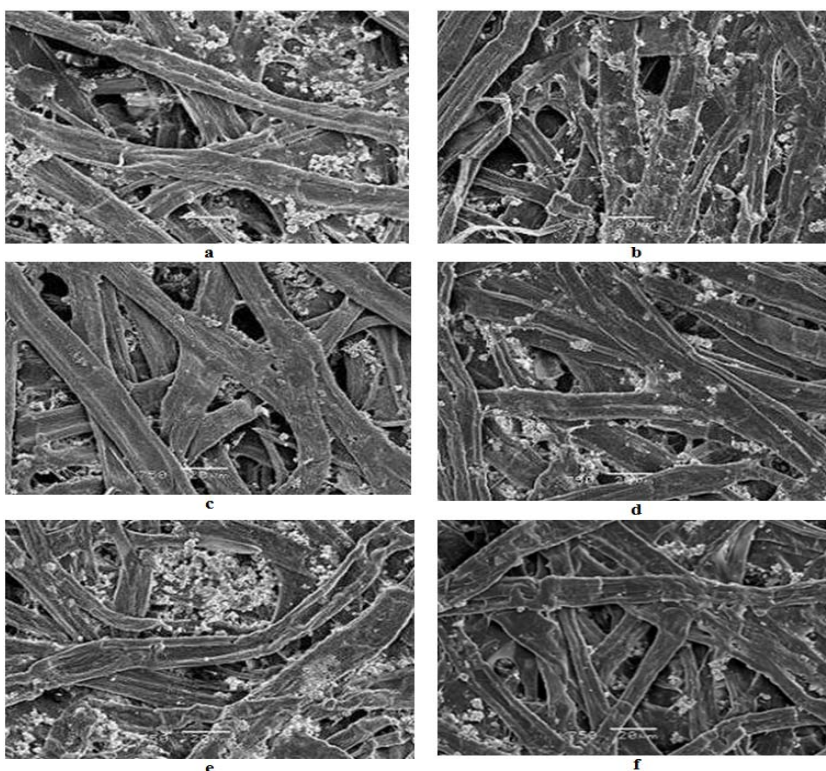


Figure 3. Observation on scanning electron microscope (SEM) to determine structural damage of the papers treated with the essential oils of clove before and after accelerated ageing. Bar is equivalent to 20 μm at 750X:

- a - paper without EO, before artificial aged; b - paper with garlic EO pure, before artificial aged;
- c - paper with clove EO pure, before artificial aged; d - paper without EO, after artificial aged,
- e - paper with garlic EO pure, after artificial aged; f - paper with clove EO pure, after artificial aged.

Table 3. Behavior of the pH and copper number of papers treated with three different concentrations of each EO before and after artificial or accelerated aged.

Concentration of the essential oil	pH			Copper number		
	BAA ± SD	AAA ± SD	Difference (AAA – BAA)	BAA ± SD	AAA ± SD	Difference (AAA – BAA)
	Garlic (50%)	7,67 ± 0,08 (e)	7,34 ± 0,11 (abc)	-0,33	1,52 ± 0,04 (h)	0,61 ± 0,04 (d)
Garlic (12.5%)	7,47 ± 0,19 (bcd)	7,25 ± 0,04 (a)	-0,22	0,76 ± 0,06 (e)	1,13 ± 0,04 (f)	0,37
Garlic (7.5%)	7,47 ± 0,03 (cd)	7,25 ± 0,14 (a)	-0,22	0,7511 ± 0,0004 (e)	0,272 ± 0,002 (a)	-0,48
Clove (50%)	8,01 ± 0,77 (f)	7,38 ± 0,22 (abc)	-0,63	1,31 ± 0,05 (g)	1,22 ± 0,04 (fg)	-0,08
Clove (12.5%)	7,30 ± 0,06 (bcd)	7,45 ± 0,18 (ab)	0,15	0,48 ± 0,02 (bc)	0,21 ± 0,03 (a)	-0,28
Clove (7.5%)	7,60 ± 0,20 (de)	7,32 ± 0,20 (abc)	-0,28	0,254 ± 0,001 (a)	0,864 ± 0,001 (e)	0,60
Control (paper without EO)	7,91 ± 0,21 (f)	7,68 ± 0,19 (e)	-0,23	0,331 ± 0,004 (b)	0,51 ± 0,13 (cd)	0,18

SD: Standard deviation; BAA: Means obtained Before Artificial Ageing or accelerated ageing; AAA: Means obtained After Artificial Ageing or accelerated ageing; (a), (b), (ab), (c), (abc), (bc), (d), (bcd), (cd), (e), (de), (f), (g), (fg), (h): Indicates significant differences according to ANOVA-1 and Multiple Range tests ($P \leq 0.05$) on comparing the effect of the EOs on papers at different concentrations before and after the artificial aged.

In relation to the copper number, indicative of the molecular damage of the cellulose, an increase of the values was obtained before accelerated ageing. The lowest value was obtained with clove oil at 7.5% followed by the solution with 12.5% concentration. Significant differences were not observed between the obtained values with clove oil at 12.5% and control (without EO), but other concentrations were markedly different. After the artificial ageing, the copper number varies significantly with a marked increase in the presence of garlic oil at 12.5% and clove oil at 50% and 7.5%. This behavior does not follow a trend directly related to the concentration of EO analyzed. However, it is likely that this harmful effect on cellulose will decrease at lower concentrations of clove.

According to Zervos [26] damaging the cellulose is inevitable with the natural or artificial ageing because the cross linking between free groups of this molecule or auto-cross linking of the cellulose chains always occurs. Therefore, the contact of the cellulose with other molecules (for example with compounds of the EOs) will inevitably cause molecular damage in the paper and this damage could be more significant or not, depending on the crystallinity of the cellulose molecule, the concentration of the other molecules and their molecular reactivity, fundamentally. However, an alternative treatment that does not cause significant damage to the paper would have to be found.

Conclusions

The obtained results demonstrated that the EOs of *Allium sativum* (garlic) and *Syzygium aromaticum* (clove) proved to be effective against fungi that cause degradation and deterioration to documentary heritage, although antifungal activity of garlic was higher than clove. Also, these EOs inhibit the biofilm formed by the fungi studied on paper and the structural characteristics of paper were not affected by the pure essential oils; therefore these products are a good alternative for the control of fungal biodegradation.

Nevertheless, we consider that some detrimental effect on the cellulose could be decreased at lower concentrations of EOs.

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