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Original article

Clotrimazole and calcium hydroxide nanoparticles: A low toxicity antifungal alternative for paper conservation

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ABSTRACT

Clotrimazole is a well-known antimycotic agent, listed in the World Health Organization List of essential medicines, with minimal health side effects acknowledged throughout a long certification period. In this study, clotrimazole in isopropanol was tested as a potential antifungal treatment for paper objects. The antifungal properties of this azole compound were evaluated against five of the most common fungal species affecting paper collections. The addition of a deacidifying agent, calcium hydroxide nanoparticles, resulted in a multipurpose formulation also aimed at neutralizing the deleterious effects of acids excreted by fungi. Clotrimazole showed antifungal activity against all tested fungal species and its effectiveness followed the ascending order: *Chaetomium globosum* < *Cladosporium cladosporioides* < *Penicillium chrysogenum* < *Aspergillus niger* < *Penicillium corylophilum*. The best relationship between minimal concentration and fungal inhibition was achieved for 0.05% clotrimazole. The impact of the tested formulation on paper preservation was evaluated in terms of pH, colour and folding endurance, using moist heat artificial ageing. Clotrimazole and calcium hydroxide nanoparticles protected the paper from acidification and loss of folding endurance in the long term, thus representing a non-aqueous alternative treatment for paper affected by fungi.

1. Introduction

Paper documents and works of art are very susceptible to fungal development due to their organic composition and hygroscopicity. Under favourable environmental conditions, such as elevated temperature and relative humidity (especially in the presence of water related disasters), fungal growth occurs. Throughout history, several toxic methods have been used to prevent and stop fungal deterioration on paper-based materials [1]. More recently, a growing concern about environmental and health issues has led to the research on new antifungal alternatives, with lower toxicity [2–6]. Nevertheless, all existent antifungal methods have strengths and weaknesses. They present different degrees of efficacy, human toxicity and secondary effects on heritage materials. Therefore, the development of non-toxic and safer methods/compounds to treat fungal biodeterioration is considered a top research priority by paper conservators [7].

In this paper, we report the potential of a well-known antimycotic agent from the pharmaceutical field, clotrimazole (CLT), as an antifungal treatment of paper collections. We intended to develop a treatment with low toxicity to humans and safe to the treated paper objects, which would also be accessible and easy to apply.

CLT is an imidazole derivative with a broad spectrum antimycotic activity that was first synthesized in the late 1960s [8] and is currently listed in the World Health Organization List of essential medicines [9]. This compound is poorly absorbed by the skin (< 0.5%) and the small amount absorbed is metabolized in the liver and excreted in bile [10].

First commercialized in the early 1970s, CLT is one of the earliest azoles used for treating cutaneous fungal infections [11]. More

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recently, CLT has also proven to have anti-malarial [12], anti-cancer [13] and neuroprotective activities [14], besides having metal corrosion inhibition properties [15].

The antifungal activity of CLT is attributed to the inhibition of ergosterol biosynthesis in microbial cell membranes, affecting cell membrane integrity and function. It also inhibits the movement of Ca^{2+} and K^{+} ions across the plasma membrane constraining cell proliferation, and interferes with Ca^{2+} binding and Ca^{2+}-dependent cellular processes [16].

The unremarkable health side effects of this compound tested throughout a long certification period [8], together with its chemical and physical properties compatible with paper conservation, such as a white colour in the solid state [17], radical scavenging properties [18], and a weak base nature [12], have made CLT a good candidate for use in paper artefacts.

With the aim of preparing a multipurpose formulation that would simultaneously inhibit fungal development and neutralize the deleterious effects of already excreted acidic fungal metabolites, the addition of calcium hydroxide to CLT was also tested. The presence of an alkaline compound in this formulation could also prevent the chemical decomposition of CLT, since this compound is stable in the alkaline environment but undergoes decomposition in acidic solutions [19].

Calcium hydroxide is one of the most used deacidification compounds in paper conservation [20] due to the long-term physicochemical stability between calcium and cellulose [21]. This compound when exposed to air forms calcium carbonate, which works as an alkaline reserve. Furthermore, calcium hydroxide has also been shown to inhibit fungal growth on paper samples when used as a treatment for paper deacidification [22].

As clotrimazole is poorly water soluble and $Ca(OH)_2$ is insoluble in alcohols, a suspension of $Ca(OH)_2$ nanoparticles (NPs) in isopropanol was used in the tested formulation. Isopropanol is also the commonly used solvent in CLT antimycotic spray formulations [23]. The suspension of $Ca(OH)_2$ NPs in isopropanol has already been studied extensively and has proven to have good deacidification properties [24,25]. The smaller size of $Ca(OH)_2$ NPs compared to the commercial micrometric powder form allows for a better penetration of the paper's fibre net. Since the specific surface area of a solid exponentially increases with the decrease of its volume, these nanoparticles also have a much greater surface area of the alkaline compound available to react, resulting in a superior capacity for neutralization.

The effectiveness of CLT was firstly evaluated by determining the minimum concentration for fungal inhibition on five of the most commonly identified fungal species on paper collections: *Aspergillus niger*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Penicillium chrysogenum* and *Penicillium corylophilum* [26–29]. Besides being tested individually, the fungal species were also tested together as mixed inoculum to simulate a real case scenario, where paper collections are exposed to contamination from several species. In those conditions, competition and suppression strategies can occur, and only the most well adapted organism(s) will develop. The determined minimal CLT concentration for fungal inhibition was afterwards tested mixed with calcium hydroxide nanoparticles as an antifungal and deacidifying treatment for paper samples.

To ascertain the safeness of the tested treatment for application on paper-based cultural heritage, the short- and long-term effects of each one of the tested compounds, on the chemical and physical properties of paper (colour, pH and folding endurance) were evaluated using moist heat artificial ageing. To the best of our knowledge, this is the first evaluation study of CLT and $Ca(OH)_2$ nanoparticles against fungal biodeterioration on a paper substrate.

2. Materials and methods

2.1. Paper

Whatman filter paper #1 was selected as the model paper due to its additive-free, high pure cellulose content (98% w/w), which reduces the number of variables in the results, and also due to the predominance of its use in paper conservation and biodeterioration research [30,31], allowing for a comparison with other studies. This paper has an average thickness of 180 μm, a grammage of 88 g/m^2 and 0.06% ash content.

2.2. Synthesis of $Ca(OH)_2$ nanoparticles

The synthesis of $Ca(OH)_2$ was performed according to the literature [32,33]. Sodium hydroxide (NaOH, CAS No. 1310-73-2Akzo Nobel, Netherlands), calcium chloride dihydrate, ($CaCl_2{\cdot}2H_2O$, CAS No. 10035-04-8, Sigma-Aldrich, USA) and isopropanol (C_3H_8O, CAS Number 67-63-0, ≥ 99.8%, Sigma-Aldrich) were used without further purification.

Two separate aqueous solutions containing 0.8 M of NaOH and 0.4 M of $CaCl_2$, respectively, were heated and maintained at 90 °C ± 5 °C. The NaOH solution was then rapidly added to the $CaCl_2$ solution. The $Ca(OH)_2$ precipitate was left to settle under a nitrogen atmosphere to avoid carbonatation and the supernatant discarded. The remaining suspension was washed repeatedly with distilled water to remove the NaCl by-product. The removal of chlorine was controlled using the Mohr method [34]. The paste resulting from the last centrifugation was concentrated at 40 °C under moderate vacuum using a Büchi Glass oven B-580 C, until a weight ratio $Ca(OH)_2/H_2O$ of 0.8 [24] was achieved. The paste was dispersed in isopropyl alcohol, achieving a $Ca(OH)_2$ concentration of 4.3 g/L [24].

Before mixing with the antifungal solution, the NPs suspension was further diluted in isopropanol to a 0.86 g/L $Ca(OH)_2$ concentration. This was the concentration that according to a previous study [25] raised the pH of an early 20th century paper to c. 8.30 (within the safe range of 8–9), avoiding alkaline deterioration [35].

The size of the obtained particles was analysed by Field Emission Gun Scanning Electron Microscope (FEG-SEM, JEOL 7001F) and Dynamic Light Scattering (DLS, Horiba Scientific, nanoparticle sizer SZ-100).

The composition of the particles was analysed by X-ray diffraction (XRD) performed with a PANalytical X'Pert PRO MPD X-ray diffractometer equipped with X'Celerator detector, with a Cu Kα radiation source (wavelength 1.540598 Å).

2.3. Tested formulations

Antifungal formulations were prepared by diluting clotrimazole (CLT, CAS No. 23593-75-1, Alfa Aesar, USA) in isopropanol in the following w/v concentrations: 0.001%; 0.01%; 0.05%; 0.1%; 0.5%; 1%; 2.5%.

The CLT formulation presenting the best antifungal results vs. minimum concentration was then tested mixed with a dispersion of $Ca(OH)_2$ nanoparticles (CLT + NPs). The mixture was prepared by diluting the respective CLT quantity in the NPs dispersion in isopropanol, to attain the selected concentration.

2.4. Inoculum preparation

C. globosum Kunze, *C. cladosporioides* (Fresen) G.A. de Vries, and *P. chrysogenum* Thom were obtained from the mycological collection of Universidade do Minho (Braga, Portugal). *A. niger* van Tieghem was obtained from the mycological collection of

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