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Interferometry as a tool for evaluating effects of antimicrobial doses on *Mycobacterium bovis* growth

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SUMMARY

Interferometry was used together with the conventional microplate resazurin assay to evaluate the antimycobacterial properties of essential oil (EO) from fruits of *Pterodon emarginatus* and also of rifampicin against *Mycobacterium bovis*. The aim of this work is not only to investigate the potential antimycobacterial activity of this EO, but also to test the interferometric method in comparison with the conventional one. The Minimum Inhibitory Concentration (MIC) values of EO (625 μg/mL) and rifampicin (4 ng/mL) were firstly identified with the microplate method. These values were used as parameters in Drug Susceptibility Tests (DST) with interferometry. The interferometry confirmed the MIC value of EO identified with microplate and revealed a bacteriostatic behavior for this concentration. At 2500 μg/mL interferometry revealed bactericidal activity of the EO. Mycobacterial growth was detected with interferometry at 4 ng/mL of rifampicin and even at higher concentrations. One important difference is that the interferometric method preserves the sample, so that after weeks of quantitative observation, the sample can be used to evaluate the bactericidal activity of the tested drug.

1. Introduction

Tuberculosis is a disease of global significance, which has presented a large number of strains resistant to tuberculostatic drugs [1]. The etiologic agents are mycobacteria belonging to the *Mycobacterium tuberculosis* complex [2].

The gene *rpoB* of the *M. tuberculosis* complex species is the target of rifampicin. This drug inhibits the transcriptions and kills bacteria [3]. The occurrence of mutations in *rpoB* permits the replication of the bacilli, reducing the chances of curing the patient [4]. Thus, the early identification of resistant individuals from samples of sputum or from susceptible standard strains is fundamental not only in order to optimize the cure strategy, but also to help the investigation of new antimycobacterial drugs.

Among the species that cause tuberculosis, *Mycobacterium bovis* is often used as a model to investigate substances with potential antimycobacterial activity [5].

The slow growth of mycobacteria related to tuberculosis has led several research groups to develop technologies more sensitive to mycobacterial growth, for instance the use of the microdilution

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technique with substances revealing viability [6]. This technology optimizes the diagnosis, the detection of strains resistant to drugs and also allows investigations about sources of new antimycobacterial substances. The search for antimycobacterial products from plants predominantly used the susceptibility tests of microplate with resazurin, MTT or Alamar blue [6,7].

Several optical techniques have been developed to identify bacteria species, and to evaluate their presence, their growth rate, and/or drug susceptibility. For instance, bacterial growth was detected using the elastic light scatter patterns, the optical forward-scattering, the Fourier spectra, the degeneration of Fraunhofer diffraction and the Fresnel diffraction patterns [8–13]. Any of these methods, when coupled with a Mach-Zehnder interferometer, has been able to differentiate the patterns of colonies formed as a function of time [12]. This association provides an identification of the bacteria present in the cultures under investigation, that improves their diagnostic processing and also adds the possibility of using this method in DST with bacteria that do not belong to the slow growth mycobacteria group.

A LASER interferometric method, using Mach-Zehnder interferometer, was used for detecting the saponin interactions with *Proteus mirabilis* [14]. The bio-layer interferometry was used to evaluate the affinity of complement system molecules with the *M. tuberculosis* molecules [15] and for detection of *M. tuberculosis* from the sputum samples [16].

In a previous study a Michelson interferometer was used to determine the number of mycobacteria (*M. bovis*-BCG) in samples with liquid culture medium as a function of time [17]. This interferometric method measures the refractive index changes of the culture medium caused by mycobacterial metabolism without any use of chemical reagents such as antibodies or any indicators. Therefore the method preserves the bacterial sample and permits real time observation during long periods of time as well as future usage of the samples.

The association of Michelson interferometer technique with microplate technique allowed the detection of antimycobacterial activity in the hexane extract from fruits of *Pterodon emarginatus* (Vogel) [18]. Moreover, this technology was able to provide evidence of the emergence of resistant strains in the sample. This occurred with both rifampicin and plant material [17,18].

The *P. emarginatus* is a Brazilian native species that belongs to the Fabaceae family and occurs in the Brazilian cerrado. It presents synonyms as *Pterodon pubescens* and *Pterodon polygalaeflorus* and is popularly known as white Sucupira [19]. The bioactivity of this species has been evaluated in several studies (Table 1) and its relevance as a medicinal plant has been proven [20]. Consequently, new pharmacological possibilities have also arisen.

The fruits of *P. emarginatus* are often used in traditional medicine for the treatment of rheumatism and infections of the throat [44,45]. Such activities practiced by the population of the cerrado stimulated the consumption of these fruits in markets and pharmacies of natural products, by persons residing in places that do not have specimens of *P. emarginatus*. Among the bioactivities most investigated about this species are its anti-inflammatory and antinociceptive potential, as shown in Table 1. Investigations about its bioactivity as a potential antimycobacterial agent are relevant in order to contribute to finding new pharmacological possibilities against tuberculosis.

In order to investigate the potential use of interferometry as an auxiliary tool in research for antimycobacterial substances, this study presents the results obtained from the comparison of two techniques for detection of mycobacterial growth under the action of the essential oil from fruits of *P. emarginatus* and under the action of rifampicin, a standard antibiotic for treating tuberculosis.

2. Materials and methods

2.1. Plant material

The fruits of *P. emarginatus* were purchased in natural product pharmacies of Juiz de Fora, Minas Gerais, Brazil. The essential oil was obtained from the fruits belonging to the same batch of fruit used in our recent study [18].

2.2. Identification of plant material

Herborization, the preparation of a voucher specimen and deposit in herbarium, were not possible because the specimens were obtained in a pharmacy of natural products, which are not accompanied by samples of their aerial parts (Figure 1). However, as in the work by Nunes et al. (2003) [46], identification based on the literature was possible from samples obtained in markets with healers. The purchase of the material was performed by asking for “Sementes de Sucupira”. The term “Sementes de Sucupira”, or simply Sucupira, is the term used by people to get the product in pharmacies or health food markets. The plant material bought came wrapped in a plastic package, with labels referring to the product as Sucupira seed (*Bowdichia virgilioides*) (Figure 1A). Through taxonomic key by Filardi et al. (2007) [47] associated with the morphological study of fruits and seeds of *P. emarginatus* developed by Pinto et al. (2014) [48], it was possible to identify the species of plant material purchased as *Pterodon emarginatus*. As described by Filardi et al. (2007) [47] and Pinto et al. (2014) [48], the fruit obtained was a cryptosamara (Figure 1D) without fins. Its seeds coincided morphologically with the seeds of *P. emarginatus*

Table 1
Studies of bioactivities of *Pterodon emarginatus*.

Bioactivity investigated	Author of publication
Chemoprophylactic agent in schistosomiasis	Mors et al. (1967) [21]; Santos Filho et al. (1987) [22]
Anti-inflammatory activity	Alves (2012) [23]; Cardoso et al. (2008) [24]; Carvalho et al. (1999) [25]; Dutra et al. (2009) [26]; Galceran et al. (2011) [27]; Hoscheid et al. (2013) [28]; Moraes et al. (2012) [29]; Sabino et al. (1999) [30]
Antinociceptive activity	Alves (2012) [23]; Galceran et al. (2011) [27]; Coelho et al. (2005) [31]; Negri, Mattei and Mendes (2014) [32], Nucci et al. (2012) [33]; Pereira et al. (2011) [34]; Spindola et al. (2010) [35]; Spindola et al. (2011) [36]
Protective action against oxidative stress	Paula et al. (2005) [37]
Larvicidal activity	Pimenta et al. (2006) [38]
Antimicrobial	Alves (2012) [23]; Machado et al. (2012) [18]; Menna-Barreto et al. (2008) [39]
Antitumor	Dutra et al. (2012) [19]; Pereira et al. (2010) [40]; Pereira et al. (2012) [41]
Anticoagulant	Calixto et al. (2007) [42]
Gastroprotective activity	Rozza and Pellizzon (2013) [43]