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Interferometry as a tool for the susceptibility test to evaluate the activity of plant derivative against rapidly growing mycobacteria

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Abstract
The susceptibility tests applied in the assays that investigate the potential of plant derivatives to be used as source of medicine against mycobacteria are often performed by using the broth microdilution method in microtiter plates. A quantitative interferometric method has been used to investigate the susceptibility of slowly growing mycobacteria. In the present article, this interferometric method is applied to rapidly growing mycobacteria. Samples of *Mycobacterium smegmatis* were exposed to methanol extract of fruits from *Stifftia chrysantha* and in this case, the interferometric method also complemented the conventional method by adding quantitative information.

Keywords: susceptibility tests, *Stifftia chrysantha*, *Mycobacterium smegmatis*, interferometry

Introduction
Background
Researches looking for new antimicrobial sources are continuously needed, once the bacteria are able to acquire the resistance to the standard antimicrobials in a continuous way (Nguyen, 2016) [11].

In general, the studies that investigate plants as a potential source of medicine against mycobacteria use the broth microdilution method for the Minimal Inhibitory Concentration (MIC) detection (Balouiri *et al*., 2016) [2]. However the readout of this method is rather qualitative and this turns it difficult to establish standard values of susceptibility especially when this qualitative judgment is applied to plant material with considerable variability of composition.

Some studies evaluated interferometry as a method to detect the mycobacterial growth. This method provides quantitative results and is able to detect the growth of a small number of bacteria. The first of them showed that the interferometry was able to follow the growth of *Mycobacterium smegmatis* and *Mycobacterium bovis* (Machado *et al*., 2008) [6].

The investigations using the interferometry as a tool to evaluate the activity of plant derivative against mycobacteria have already been conducted using a slowly growing mycobacteria species, the *M. bovis* (Machado *et al*., 2012, 2013, 2015a) [7, 8, 9]. However, the interferometric method had not been used for susceptibility tests of rapidly growing mycobacteria.

The *M. smegmatis* is a rapidly growing mycobacteria of low pathogenicity and it is present in a few cases of infection (Best & Best, 2009). This species can be used as a model in researches for new therapeutic products against diseases as mycobacteriosis and tuberculosis, which are caused by virulent mycobacteria (Altaf *et al*., 2010) [1].

Stifftia chrysantha J. C. (Mikan) is a plant that belongs to the Asteraceae family. This plant is generally used as ornamental, due to their flowers. Oliveira *et al*. (1999) [12] identified some flavonoids in this species. This chemical class of substances is described as a potential component that presents antimicrobial agent (Simões *et al*., 2010) [13]. Marques *et al*. (2013) [10] showed some antileishmanial and antimycobacterial activities using different extracts of this plant.

The aim of the present work is to evaluate the use of interferometry as a tool to characterize the susceptibility of *M. smegmatis* to the methanol extract from fruits of *S. chrysantha*. Since the interferometric method has been designed especially for slowly growing mycobacteria, the success of tests with *M. smegmatis* was a nontrivial question.

Materials and Methods

Obtaining the plant material

Aerial parts of plant (flowers, fruits) of *S. chrysantha* were collected under the supervision of botanist Dr. Roberto L. Esteves in the garden of National Museum of Rio de Janeiro. The voucher code is R208153. Then, the fruits were separated from the flowers. The fruits (40g) were used to obtain the methanol extract. Firstly, the fruits were dried at room temperature (approximately 25° C) and then, they were submitted to the static maceration using the methanol as solvent. This solution was concentrated through the removing of the residual solvent under reduced pressure using the rotary evaporator (Buchi). The extract obtained was kept in ambar glass at - 20°C until its use.

Suspension of mycobacterium smegmatis (ATCC 14468)

In order to use the *M. smegmatis* on its log phase of growing, it was seeded in Lowenstein-Jensen medium and after 7 days the colonies were harvested and mycobacterial suspension containing 3 x 10^8 CFU/mL was obtained though dilution. After that, this suspension was diluted to 1:25 using broth Müeller Hinton (Becton Dickinson and Company) and this suspension was used for the susceptibility tests.

Susceptibility test using broth microdilution method

The broth microdilution susceptibility test was conducted using 96-wells microtiter plates according to Machado *et al*. (2015b) [9]. One initial solution of the extract was prepared diluting the obtained methanol extract in DMSO (dimethyl-sulfoxide) 10% and subsequently in sterile water until the concentration of solution became equal to 20mg/mL. This solution was diluted in culture medium (Broth Müller-Hinton) and the final concentration was 5mg/mL and this one was used in tests. The final concentration of DMSO in the tested solutions was minor than 10% and according to our results, this concentration was not able to inhibit the mycobacterial growth. Then, the procedures of the test were done. Initially, the external wells of microtiter plates were filled with sterile water. The other wells were filled with 100µL of culture medium (Broth Müller-Hinton). In the next step, the second wells of the plate were filled with 100µL of extract solution (5mg/mL). From these wells up to the sixth, a serial dilution was done and the concentrations of the extract solution into the wells were 2.5mg/mL, 1.25mg/mL, 0.625mg/mL, 0.3125mg/mL, 0.156mg/mL and 0.0781mg/mL (the final volume in each well, at this moment, was 100 µL). From each well, 10µL of solution was removed and it was added 10µL of the suspension of *M. smegmatis*. The tested concentrations were 2.25mg/mL, 1.125mg/mL, 0.5625mg/mL, 0.28125mg/mL, 0.140625mg/mL and 0.0703125mg/mL. The plates were closed and kept at 37° C during 7 days. After this time, the growth was detected visually and the MIC was determined as the minor concentration able to inhibit the mycobacterial growth. The positive control was done using the ciprofloxacin (ALDRICH Chemistry – Lot: 17850) at the following concentrations (0.5 – 0.9 x 10-7µg/mL, considering a serial dilution 1:2 inside the wells). The negative control did not contain any extract or antibiotic, which allowed the mycobacterial growth. All assays were done in triplicate and were repeated at least three times.

The interferometer & the susceptibility test using the interferometry

The interferometer, how does it work?

The interferometry is an optical technique able to measure different things such as: distance, deformation, change of chemical composition of media and temperature changes. Also, it is able to help in different branch of science as: the Astronomy, Oceanography and Seismology (Halliday *et al*., 2014) [4].

There are different interferometers, and in this work a Michelson Interferometer was used. In an optical interferometer a light wave is divided into two beams. The two new beams follow different ways and are recombined to construct an interference figure (Figure 1). In the Michelson Interferometer the two beams are recombined at the same location of beam splitting returning to that place on the same path after a reflection. When one of the beams travels a different distance or when one of the beams takes a different time to travel a defined distance, it is possible to measure this changing with high precision.

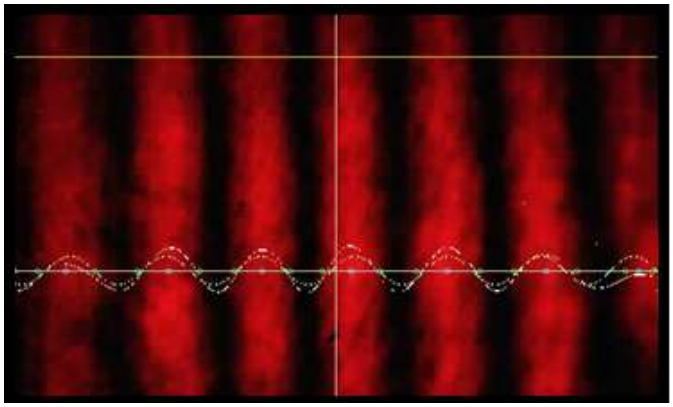

Fig 1: Interference figure obtained using the interferometry

The microrganisms use some components of the culture medium. It changes the chemical composition of the culture medium, reducing the refractive index and increasing the speed of light propagation. Finally, these changes will be detected with high precision using the interferometry.

The susceptibility test using the interferometry was done evaluating the antimicrobial activity of extract from fruits of *S. chrysantha* using an apparatus described in Machado *et al*. (2015a) [9]. The procedures are described below: firstly, the MIC value of extract was identified using broth microdilution susceptibility test. Then, the concentrations of plant extract used at the current test were: 0.070mg/mL; 0.140mg/mL and 0.280mg/mL, and all of them were prepared using the culture medium Broth Müller-Hinton as diluent. The sample-holders (Figure 2B) were filled as follows: the control group presented, on both wells, culture medium and into one of them a suspension of *M. smegmatis* (6 x 10^3 CFU/mL); the group 1 presented, on both wells, a solution of 0.070mg/mL of the plant extract and into one of them, a suspension of *M. smegmatis* (6 x 10^3 CFU/mL); the groups 2 and 3 were prepared as described for group 1, but containing the following concentrations of plant extract 0.140mg/mL and 0.280mg/mL, respectively. The contents of wells in groups 4, 5 and 6 were, for the both wells, the culture medium and the standard antibiotic (ciprofloxacin) at two different

concentrations (0.256 µg/mL, 0.128 µg/mL), respectively. As the other groups, into one of wells were added the mycobacterium suspension. The groups were assayed in triplicate.

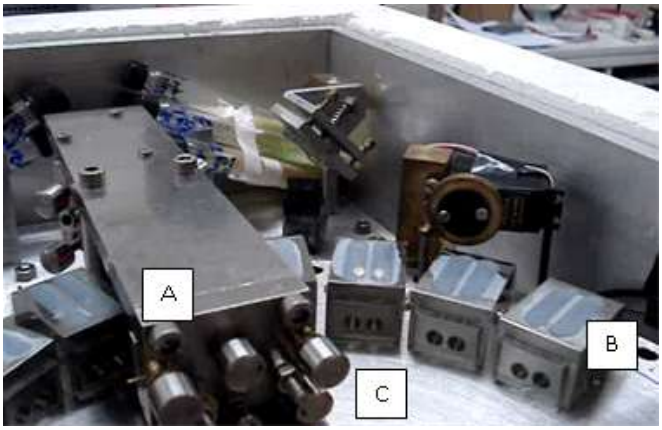

Fig 2: A) Interferometer; B) Sample-holders with two wells each one; C) The gyratory disc. The Figure 2 also shows the apparatus with sample-holders (B) above the gyratory disc (C), that is responsible for the positioning of each sample-holder into the interferometer (A). At this place, the LASER beams cross the culture medium and back to the camera, that captures the image of the interference figure (Figure 1). Then, the changes of refractive index are detected through the comparison of the current image with the last one and the growth rate is calculated

Results and Discussion

The results obtained with the broth microdilution tests showed that the methanol extract from fruits of *S. chysantha* can be considered as a potential antimycobacterial source, as the MIC value (0.14 mg/mL) was minor than 0.200 mg/mL (Tosun *et al*., 2004) [14].

The Figure 3 shows the graph that contains the relative growth rate of mycobacterium submitted to each concentration of plant extract. It can be observed that two samples (0.14 mg/mL and 0.28 mg/mL) showed a relative growth rate equal zero. All samples with ciprofloxacin had zero growth rate.

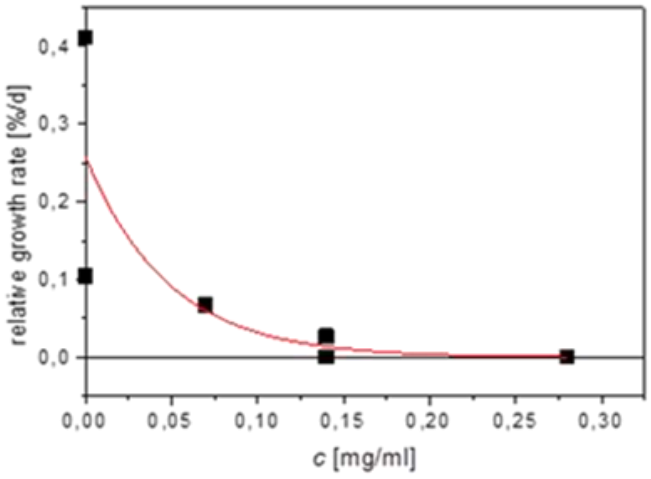

Fig 3: Effect of different concentrations of the methanol extract from fruits of *S. chrysantha* on the growth rate of *M. smegmatis.* c [mg/mL] represents the axis of the extract concentrations (0mg/mL; 0.07mg/mL; 0.14mg/mL and 0.28mg/mL. The red line represents the tendency to the reduction of the relative growth rate with the increase of extract concentration

The concentration detected as the MIC using the broth microdilution in microtiter plates was 0.14 mg/mL and one of the samples showed, to this concentration, a relative growth rate greater than zero (0.2619 %/d). This growth could be considered as a low growth, but it is necessary to highlight that some bacteria were resistant to the bactericidal effect of the extract in this concentration and this bacterial growth could produce a relevant increase of bacterial population and finally, it could lead to the failure of the treatment.

Taking into account that this concentration was the MIC when the susceptibility tests using broth microdilution in microtiter plates were done, it was possible to consider the interferometry as a tool to detect the growth of potential resistance specimens during the examination of the antimicrobial plant potential.

This way, the used tool was able to detect the growth of *M. smegmatis* population and also it showed the emergence of potential resistance of this bacteria to the tested extract. The method permitted to judge the reduction of the relative growth rate with increasing concentration of the methanol extract, quantitatively. The observed reduction of growth rate confirms the considerations published by Tosun *et al*. (2004) [14], which had been obtained with microtiter plates. On the other hand, it is necessary to highlight that the extract was not able in exterminate all samples of mycobacteria during the tests. Thus, this new readout of the mycobacterial growing can contribute to the investigations of new sources to antimycobacterial medicines using plant derivatives and the broth microdilution method, once the interferometry detects minimal bacterial growth non detected by the microtiter method of susceptibility test.

References

1. Altaf M, *et al*. Evaluation of the Mycobacterium smegmatis and BCG models for the discovery of Mycobacterium tuberculosis inhibitors. Tuberculosis (Edinburgh, Scotland). 2010; 90(6):333-337.
2. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis. 2016; 6(2):71-79.
3. Best CA, Best TJ. *Mycobacterium Smegmatis* Infection of the Hand. Hand. 2009; 4(2):165-166.
4. Halliday D, Resnick R, Walker J. Fundamentos de física: óptica e física moderna. 9. ed. Rio de Janeiro: LTC. 2014, 4.
5. Machado RRP, *et al*. Metabolic activity interferometer: description and calibration of an interferometric method to measure growth of mycobacteria. European biophysics journal. 2008; 38(1):111-119.
6. Machado RRP, *et al*. Metabolic Activity Interferometer: A Powerful Tool for Testing Antibiotics. Journal of Sensors. 2012, 7.
7. MACHADO RRP, *et al*. The effect of essential oil of Syzygium cumini on the development of granulomatous inflammation in mice. Revista Brasileira de Farmacognosia. 2013; 23(3):488-496.
8. Machado RRP, *et al*. Interferometry as a tool for evaluating effects of antimicrobial doses on Mycobacterium bovis growth. Tuberculosis (Edinburgh, Scotland). 2015a; 95(6):829-838.
9. Machado RRP, *et al*. Screening antimycobacterial activity of Baccharis dracunculifolia, Centella asiatica, Lantana camara and Pterodon emarginatus. Revista Brasileira de Plantas Medicinais. 2015b; 17(4):891-899.

10. Marques AM, *et al*. Evaluation of in vitro antileishmanial and antimycobacterial activities of Stifftia chrysantha J.C. Mikan extracts. Revista Fitos Eletrônica. 2013; 7(4):252-258.
11. Nguyen L. Antibiotic resistance mechanisms in M. tuberculosis: an update. Archives of Toxicology. 2016; 90(7):1585-1604.
12. Oliveira MCC, *et al*. Flavonóides das flores de Stiffitia chrysantha Mikan. Química Nova. 1999; 22(2):182-184.
13. SIMÕES C, *et al*. Farmacognosia: da planta ao medicamento. 6. ed. Florianópolis: UFSC, 2010.
14. Tosun F, *et al*. Antimycobacterial screening of some Turkish plants. Journal of Ethnopharmacology. 2004; 95(2-3):273-275.