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Original Paper

Anti-mycobacterial and immunomodulatory activity of *n*-hexane fraction and spathulenol from *Ocotea notata* leaves

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Abstract

Ocotea notata (Lauraceae) is popularly known as white-cinnamon. *Ocotea* species have several medicinal uses, especially for treating chest pain, rheumatism and wounds. The present study aimed to analyze the chemical composition of *O. notata n*-hexane fraction, in addition to its anti-mycobacterial and immunomodulatory activities. The *n*-hexane fraction was analyzed by GC-MS and was chromatographed to afford 15 subfractions (SF1-15), where SF5 was identified, by GC-MS and NMR, as the sesquiterpene spathulenol. The *n*-hexane fraction was the most potent in inhibiting nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) production on LPS-stimulated macrophages (IC_{50} 8.3 ± 0.9 and 5.9 ± 1.0 μg/mL, respectively). SF4, a major subfraction, that presents a spathulenol analogous as a constituent, also inhibited NO and TNF-α production. Spathulenol only modulated NO production (IC_{50} 45.6 ± 1.4 μg/mL). The *n*-hexane fraction, SF4, and spathulenol revealed antimycobacterial activity against *Mycobacterium bovis* BCG, *M. tuberculosis* H37Rv, and M299 strains. Spathulenol inhibited the growth of Mtb H37Rv with MIC_{50} 36.9 ± 1.5 μg/mL (167.5 ± 6.8 μM), and Mtb M299 with MIC_{50} 42.1 ± 0.5 μg/mL (191.0 ± 2.2 μM). This is the first report describing the isolation of spathulenol from *O. notata* leaves and its anti-mycobacterial activity.

Key words: antiinflammatory, anti-mycobacterial, *Ocotea notata*, Sesquiterpenes.

Resumo

Ocotea notata (Lauraceae) é popularmente conhecida como canela-branca. As espécies do gênero *Ocotea* têm vários usos medicinais, especialmente no tratamento de dores no peito, reumatismo e feridas. O presente estudo teve como objetivo analisar a composição química da fração *n*-hexano de *O. notata*, além de suas atividades antimicobacteriana e imunomoduladora. A fração *n*-hexano foi analisada por GC-MS e fracionada fornecendo 15 subfrações (SF1-15), onde SF5 foi identificado, por GC-MS e RMN, como o sesquiterpeno espatulenol. A fração *n*-hexano foi a mais potente na inibição da produção de óxido nítrico (NO) e do fator de necrose tumoral-alfa (TNF-α) em macrófagos estimulados por LPS (IC50 8,3 ± 0,9 e 5,9 ± 1,0 μg/mL, respectivamente). SF4, uma subfração majoritária, que apresenta um análogo do espatulenol como constituinte, também inibiu a produção de NO e TNF-α. O espatulenol modulou apenas a produção de NO (IC50 45,6 ± 1,4 μg/mL). A fração n-hexano, SF4 e espatulenol apresentaram atividade antimicobacteriana contra as cepas de *Mycobacterium bovis* BCG, *M. tuberculosis* (Mtb) H37Rv e M299. O espatulenol inibiu o crescimento de Mtb H37Rv com MIC50 36,9 ± 1,5 μg/mL (167,5 ± 6,8 μM) e Mtb M299 com MIC50 42,1 ± 0,5 μg/mL (191,0 ± 2,2 μM). Este é o primeiro relato que descreve o isolamento do espatulenol das folhas de *O. notata* e sua atividade antimicobacteriana.

Palavras-chave: anti-inflamatório, antimicobacteriano, *Ocotea notata*, Sesquiterpenos.

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Introduction

Tuberculosis (TB) is a major cause of death worldwide aggravated by the emergence of *Mycobacterium tuberculosis* (Mtb) multidrug-resistant (MDR-TB) strains. The World Health Organization reported 10 million new TB cases in 2017, with a high mortality rate in developing countries (WHO 2018). The long treatment duration of at least 6 months and complex regimens which involve expensive and toxic drugs hinder improvement in therapy outcomes (Dartois 2014).

Uncomplicated drug-sensitive pulmonary TB treatment is based on the use of multiple antibiotics. However, severe destructive and disseminated forms of TB such as meningitis and tuberculosis pericarditis require anti-inflammatory adjunct therapy to prevent excessive inflammation (Zumla *et al*. 2014).

The search for new molecules with dual anti-inflammatory and anti-mycobacterial activity has been encouraged for severe pulmonary TB therapy and 70% of the antibiotics in use are natural products or are derived from them (Singh & Macdonald 2010).

Ocotea notata (Nees & Mart.) Mez is a medium-sized tree belonging to the Lauraceae family, popularly known as white-cinnamon. Within the family, *Ocotea* genus presents the highest number of medicinal species, being used to treat chest pain, rheumatism, and cutaneous wounds, among others (Marques 2001; Neto & Morais 2003).

Species of the *Ocotea* genus have several activities reported in the literature such as anti-inflammatory (Zschocke *et al*. 2000; Madubanya *et al*. 2005), antioxidant (Bruni *et al*. 2004; Guerrini *et al*. 2006), antiprotozoal (Fournet *et al*. 2007), anti-allergic (Serra *et al*. 1997), central nervous system depressant (Pachú *et al*. 1993), antimicrobial (Bruni *et al*. 2004; Souza *et al*. 2004) and anti-herpetic (Dias *et al*. 2003), and have been studied for their diversity in secondary metabolites such as alkaloids, neolignans, lignans, terpenes, and flavonoids (Dias *et al*. 2003; Zanin & Lordello 2007; Barbosa-Filho *et al*. 2008; Funasaki *et al*. 2009; Cuca *et al*. 2009; Garrett *et al*. 2012).

We had previously shown that the crude ethanol extract of *Ocotea notata* leaves presented anti-mycobacterial activity and NO production inhibitory effect on LPS-stimulated RAW 264.7 macrophages. Moreover, two isolated flavonoids from ethyl acetate fraction, kaempferol-3-*O*-*β*-L-ramnopyranoside (afzelin), and quercetin-3-*O*-*β*-D-glucopyranoside (isoquercitrin) were shown to contribute to suppressing NO production, although they did not inhibit mycobacterial growth (Costa *et al.* 2015).

Thus, the aim of the present study was to continue our efforts in the study of *Ocotea notata*, collected in the *Restinga* of Jurubatiba (Rio de Janeiro state, Brazil). Specifically, to investigate the chemical composition of its *n*-hexane fraction, associated with anti-mycobacterial and immunomodulatory activities. The *n*-hexane fraction stood out as an anti-mycobacterial promising fraction and was not studied before.

Materials and Methods

Plant material

Leaves of *Ocotea notata* were collected in the early morning, on a sunny day, at Parque Nacional da Restinga de Jurubatiba, Quissamã, Rio de Janeiro, Brazil, under legal authorization (SISBIO 39673-2, SisGenAAA989F) at March 2011. A voucher specimen (RFA38751) was deposited at the herbarium of the Universidad Federal do Rio de Janeiro (Brazil).

Extraction and isolation

Fresh *O. notata* leaves (1.3 kg) were triturated and extracted exhaustedly with ethanol ACS at room temperature (crude extract). An aliquot (60.0 g) of the dried extract (78.1 g) was resuspended with methanol and partitioned with *n*-hexane to obtain the *n*-hexane fraction (26.7 g). An aliquot of *n*-hexane fraction (1.5 g) was chromatographed and submitted to column chromatography on a Si gel 60 (40-63 microns) (SILICYCLE®) (60 cm in height and 2.7 cm in diameter) and then eluted with an increasing gradient varying in between *n*-hexane/AcOEt and MeOH (0–100%) in steps of 10% (40 mL each), and separated into 15 subfractions on the basis of TLC analyses revealed with vanillin followed by sulfuric acid. The evaluation of the biological properties was restricted to *n*-hexane fraction, and subfractions SF2, SF4, SF5, SF7, and SF15 due to their amount, solubility and toxicity.

Subfraction 5, (SF5, 11 mg, eluted with EtOAc/Hex, 9:1) was identified as a pure compound by TLC and was submitted to NMR and GC-MS analyses.

Analysis by gas chromatography coupled to mass spectrometry

Fraction, subfraction, and isolated compound were submitted to GC-MS performed on Shimadzu GC-MS 2010 equipment using capillary columns of fused silica RTX-5MS (30 m × 0.25 µm) (Restek Corporation Pennsylvania, USA). The column temperature was kept at 60 °C for 1 min and was subsequently increased to 280 °C at a rate of 15 °C/min and held for 10 min. Helium was used as carrier gas with a flow rate of 1.1 mL/min. One milligram of the SF5 pure fraction was dissolved in *n*-hexane and 1 µL was injected with the aid of an autosampler. Chromatogram peaks were tentatively identified by comparing their mass spectra with those of the National Institute of Standards and Technology (NIST) library and those reported in the literature (Adams 2007). All hits that presented similarity ≥ 90% were considered and checked manually. In addition, the identity of spathulenol was confirmed by comparison of its retention time and full scan mass spectrum with the isolated sample. Furthermore, sub-fractions were compared to *n*-hexane fraction due to their chromatograms, considering peaks retention time and full scan mass spectrum.

Nuclear magnetic resonance

The isolated spathulenol compound was analyzed by mono and two-dimensional ^{1}H and ^{13}C NMR spectra and recorded on a Bruker-DRX-400 NMR spectrometer (^{1}H: 400 MHz; ^{13}C: 100 MHz) of the Instituto de Pesquisas de Produtos Naturais Walter Mors (IPPN/UFRJ).

Mycobacterial culture and antimycobacterial activity

Three mycobacterial strains differing in virulence were used in this study: an avirulent *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), Moreau vaccine strain, and two *Mycobacterium tuberculosis* strains (low virulent laboratory strain H37Rv, ATCC 27294, and highly virulent Mtb Beijing strain M299, isolated from a TB patient in Mozambique) evaluated for virulence in a previous study (Ribeiro *et al*. 2014). Mycobacterial strains were grown in suspension in 7H9 Middlebrook broth containing 10% albumin dextrose complex (ADC) and 0.05% Tween-80 at 37 °C under Biosecurity level 3 containment conditions. The hexane fraction, SF2, SF4, SF7, SF15, and spathulenol were evaluated for their antimycobacterial activity using an MTT assay (tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazole, Sigma Aldrich®) in a 96-well plate. During the logarithmic growth phase, 50 µL of bacterial suspensions were plated at 1×10^6 CFU/well and incubated with 50 µL of samples at concentrations of 4 to 500 µg/mL. The sealed plate was incubated at 37 °C and 5% CO_2 for 7 days for Mbv BCG and 5 days for the other strains. After this period, the bacterial culture was then incubated for 3 h with 10 µL of tetrazolium salt (5 mg/mL in sterile phosphate-buffered saline [PBS]) and then 100 µL of lyses buffer (20% w/v SDS/50% DMF - dimethylformamide in distilled water - pH 4.7) were added (Gomez-Flores *et al*. 1995). The plate was incubated overnight, and the reading was made using a spectrophotometer at 570 nm. A bacterial culture treated with antibiotic rifampin (Sigma Aldrich®, 95% purity) was used as a positive control, while an untreated bacterial suspension was used as a negative control.

Cell culture, treatments, and quantification of Nitric Oxide and TNF-α production

RAW 264.7 cells obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) were cultured in Dulbecco Modified Eagle medium (DMEM-F12) with 10% fetal bovine serum and gentamicin (50 µg/mL) in the presence of 5% CO_2 at 37 °C. The cells were seeded in 96--well plates (1×10^5 cells/well) in the presence or absence of samples (4 to 500 µg/mL) and/or lipopolysaccharide (LPS-*Escherichia coli* 055:B5; SigmaAldrich). A NO inhibitor (*N*G-methyl-L-arginine acetate salt - L-*N*MMA, Sigma Aldrich®, 98% purity) was used as a positive control of NO inhibition at 20 µg/mL (32.7 ± 0.9 µM/reducing NO production by 52.5 ± 1.3%) in the experiments. Culture supernatants were collected for NO and TNF-α assays after 24 h. The nitrite concentration, a stable NO metabolite, was determined using the Griess method (Park *et al*. 2009). TNF-α was measured by an L929 fibroblast bioassay, based on the sensitivity of L929 cells to the cytotoxic effect of TNF-α. To do so, the L929 cells were seeded in 96-well plates (2×10^5 cells/well). The resulting cell monolayers were treated with the macrophage culture supernatants in the presence of actinomycin D (2 µg/mL) after 24 h of incubation. Then, after 24 h of additional incubation, the viability of the L929 cells was assayed by the MTT assay (Mosmann

1983). The cytokine concentration was determined using a recombinant mouse cytokine to obtain a standard curve to correlate cellular viability and TNF-α concentration.

Macrophage cytotoxicity assay

Cytotoxic effects of samples on RAW 264.7 cell viability in cultures stimulated with LPS were determined using the MTT assay (Mosmann 1983). A cell culture was obtained as previously described and evaluated using tetrazolium salt MTT. The optical density was measured at 570 nm employing a microplate reader after incubation for 2 h with MTT solution (5 mg/ml in sterile PBS). Cytotoxicity was calculated by subtracting the ratio of the mean absorbance value for treated cells from the mean absorbance value for non-treated cells. The cytotoxicity percentage was calculated in relation to the negative control (untreated macrophages) and stimulated, and to the positive control (stimulated macrophages) culture treated with 1% (v/v) Triton X-100. Final concentrations of dimethyl sulfoxide (DMSO) were used as the solvent of the samples and were tested in parallel as a control.

Statistical analysis

The tests were performed in triplicate and values were reported as the mean ± standard error of the mean (SEM). Statistical analyses were performed by one-way ANOVA, followed by Tukey post-test, employing GraphPad Prism 4 software. The outcomes were considered significant for $p < 0.05$. The IC_{50} and MIC_{50} values were calculated by non-linear regression.

Results and Discussion

The phytochemical profile of the *n*-hexane fraction from *O. notata* extract and its subfractions were investigated in the present study. Furthermore, their anti-mycobacterial and immunomodulatory potential and cytotoxicity were evaluated, as well as for the isolated sesquiterpene spathulenol.

The permeability of mycobacteria to compounds is controlled by an outer lipid barrier based on a monolayer of characteristic mycolic acids and a capsule-like coat of polysaccharide and protein. The mycolate layer prevents entry of small hydrophilic molecules, which obtain access to the cell by way of pore-forming proteins resembling porins of Gram-negative bacteria. More lipophilic molecules can diffuse through the lipid layer (Draper 1998). The mycobacterial cell wall has a high permeability to hydrophobic compounds (Lee *et al*. 2013), which encourages investigation of the *n*-hexane fraction, a non-polar, commonly composed by terpenes, sterols and fatty acids (Cantrell *et al*. 2011).

GC-MS analyses were conducted in order to identify putative active compounds present within the *O. notata n*-hexane fraction. Then, 11 derivatives were identified in comparison to NIST library and to literature data (Adams 2007). The major components identified in the *n*-hexane fraction of *O. notata* leaves were the terpenes: (*Z*)-*epi*-*β*-santalol (17.98%), (*Z*)-*α*-santalol (10.27%), caryophyllene oxide (8.19%) and spathulenol (10.79%), with a similarity of 94, 90, 92 and 93%, respectively, to the NIST library; and in accordance to the literature data (Adams 2007). Table 1 presents the proposed identity for *n*-hexane fraction compounds.

The essential oil of *O. notata* leaves was analyzed in previous studies by GC-MS and 12 compounds were identified, accounting for 83.3% of the total components present in the essential oil. Germacrene A (22.7%) and *β*-caryophyllene (22.9%) sesquiterpenes and *α*-pinene, *β*-pinene, and terpinolene (8.7, 6.9 and 5.5%, respectively) monoterpenes were the main metabolites identified. The brine shrimp (*Artemia salina*) lethality test was performed and showed high toxicity profile for this oil with an LC_{50} value of 2.4 µg/ml (Garret *et al*. 2010).

The chemical composition of extracts, fractions, and essential oil could vary among species, in the same species and in different plant parts. Several factors influence the production of secondary metabolites, such as salinity, temperature, harvest season, soil quality, and other factors (Gobbo-Neto & Lopes 2007).

Then, SF2, SF4, and SF5, which are subfractions obtained from *n*-hexane fraction, were analyzed by GC-MS because they were the most active samples, considering anti-mycobacterial and immunomodulatory assays, as could be seen below. For subfractions, in addition to NIST information, GC-MS data were also analyzed considering the comparison of retention time and mass spectrum with those obtained for the *n*-hexane fraction. The major identified constituents in these subfractions were SF2: (*Z*)-*α*-santalol (11.74%) and spathulenol (12.81%), in Table 2; SF4: aromadendrane-type sesquiterpenoid (37%), in Table 3. SF5 presented the highest degree of purity. It was analyzed by GC-MS and sesquiterpene spathulenol (t_R 10.115

Table 1 – Chemical composition of *n*-hexane fraction from *Ocotea notata* by gas chromatography coupled with mass spectrometer GC-MS.

Component	Retention Time (t_R min)	Relative Area (%)	Compound Name	Similarity to NIST Library (%)
1	8.733	1.67	santalene	95
2	8.783	0.99	(*E*)-caryophyllene	95
3	8.858	0.57	NI	-
4	8.983	0.77	NI	-
5	9.100	0.67	NI	-
6	9.400	0.83	NI	-
7	9.467	1.37	NI	-
8	9.617	1.04	NI	-
9	9.667	1.02	NI	-
10	9.933	3.27	NI	-
11	10.458	3.27	NI	-
12	10.050	3.27	NI	-
13	10.192	10.79	spathulenol	93
14	10.258	8.19	caryophyllene oxide	92
15	10.642	1.43	NI	-
16	10.683	3.70	cadinol	90
17	10.808	7.89	NI	-
18	11.008	10.27	(*Z*)-*α*-santalol	90
19	11.128	17.98	(Z)-*epi*-*β*-santalol	94
20	11.225	5.40	bergamotol	93
21	11.342	5.73	NI	-
22	11.467	3.85	(*E*)-*β*-santalol	93
23	11.567	3.53	NI	-
24	11.692	2.50	NI	-

NI = non-identified

min), previously assigned in the chromatogram of the *n*-hexane fraction, was identified as the major volatilized component of this subfraction (99% of the relative area in GC-MS chromatogram and 90% of similarity) (Fig. 1).

The sample was submitted to ^{1}H and ^{13}C NMR spectroscopy analysis to support structural elucidation. ^{13}C NMR spectrum was decisive to the characterization of spathulenol (1) (Fig. 2), that was completed by comparison to the literature data (Inagaki &Abe 1985; Farias *et al.* 2019). The ^{13}C NMR spectrum showed fifteen carbon signals characteristic of an aromadendrane type sesquiterpene, as listed below. It is important to highlight the two carbons signals of the exocyclic double-bond, C-10 (δ 153.3 ppm) and C-15 (δ 106.3 ppm); and the one signal characteristic of a sesquiterpene alcohol (C-4; δ 81.0 ppm).

Table 2 – Chemical composition of SF2 fraction from *Ocotea notata* by gas chromatography coupled with mass spectrometer GC-MS.

Component	Retention Time (t_R min)	Relative Area (%)	Compound Name	Criteria for the Identification
1	9.98	0.77	NI	-
2	10.052	2.54	NI	-
3	10.183	12.81	spathulenol	1
4	10.385	7.51	caryophyllene oxide	1
5	10.471	2.55	NI	-
6	10.526	5.54	NI	-
7	10.564	5.54	NI	-
8	10.933	17.72	NI	-
9	11.041	11.74	(*Z*)-*α*-santalol	1
10	11.154	2.55	(*Z*)-*epi*-*β*-santalol	1
11	11.278	4.85	bergamotol	1
12	11.487	2.50	(*E*)-*β*-santalol	1
13	11.561	3.27	NI	-
14	12.380	2.44	NI	-
15	13.102	5.50	heptadecanoic acid	1, 2
16	14.272	3.74	NI	-
17	23.205	3.74	vitamin E/ α-tocoferol	1, 2

1 = Comparison of retention time and mass spectrum with those obtained for hexane fraction compounds and those of the literature (Adams 2007);
2 = Similarity with NIST library spectra (92 and 96% for compounds 15 and 17, respectively); NI = non-identified.

Spathulenol (1): Colorless oil; purity 99% (GC/MS); t_R 10.115 min; NMR data: ^{13}C NMR (100 MHz, $DMSO_{d6}$): δ ppm 53.5 (C-1), 26.7 (C-2), 41.8 (C-3), 81.0 (C-4), 54.4 (C-5), 29.9 (C-6), 27.5 (C-7), 24.8 (C-8), 38.9 (C-9), 153.3 (C-10), 20.3 (C-11), 28.7 (C-12), 16.3 (C-13), 26.1 (C-14), 106.3 (C-15).

The *n*-hexane fraction and its subfractions (SF2, SF4, SF5, SF7, and SF15) were initially evaluated for their immunomodulatory activity through evaluation of the inhibitory effect on the production of NO and TNF-α by LPS-stimulated RAW 264.7 macrophages. NO is an important chemical mediator that has several physiologic functions such as immunomodulatory action in response to many immune cells and microbicide activities during inflammatory responses. However, the increased levels of NO have a deleterious role leading to tissue damage and must be tightly regulated (Guzik *et al*. 2003; Garlanda *et al*. 2007).

The *n*-hexane fraction from *O. notata* leaves was the most potent in inhibiting NO production on LPS-stimulated macrophages when compared to control groups. Its inhibitory capacity was of 72.2 ± 1.6% at 100 µg/mL, and at 20 µg/mL inhibited 55.9 ± 1.5% of the NO production, with IC_{50} of 8.3 ± 0.9 µg/mL (Fig. 3a; Tab. 4).

Among the studied subfractions, the more pronounced inhibitory activity was presented by SF4 and followed by SF2. SF4 particularly demonstrated higher inhibitory capacity on NO production (IC_{50} 15.6 ± 1.1 µg/mL) than SF2 (IC_{50} 37.7 ± 1.1 µg/mL), although the latter inhibited 51.7 ± 1.3% of the NO production at

Table 3 – Chemical composition of SF4 fraction from *Ocotea notata* by gas chromatography coupled with mass spectrometer GC-MS.

Component	Retention Time (t_R min)	Relative Area (%)	Compound Name	Criteria for the Identification
1	12.67	37.00	aromadendrane type sesquiterpenoid	1,2
2	12.75	6.45	NI	-
3	12.88	6.35	NI	-
4	13.42	4.30	NI	-
5	14.07	4.00	NI	-
6	14.24	3.95	NI	-
7	15.00	2.90	NI	-
8	15.68	35.00	NI	-

1 = Comparison of retention time and mass spectrum with those obtained for hexane fraction compounds, and those of the literature (Adams 2007);
2 = Similarity with NIST library (94% for compound 1); NI = non-identified.

the 100 µg/mL. The SF15 and SF7 subfractions showed the worst profiles (higher IC_{50} values). Spathulenol (SF5) inhibited 50.9 ± 1.0% of NO production at 100 µg/mL, but the same was not observed in lower concentrations (20 and 4 µg/mL).

The spathulenol IC_{50} for NO inhibitory activity was calculated as 45.6 ± 1.4 µg/mL, equivalent to 206.0 ± 4.5 µM (Fig. 3a; Tab. 4).

When compared to L-*N*MMA, a known NO inhibitor used as a positive control (NO inhibition

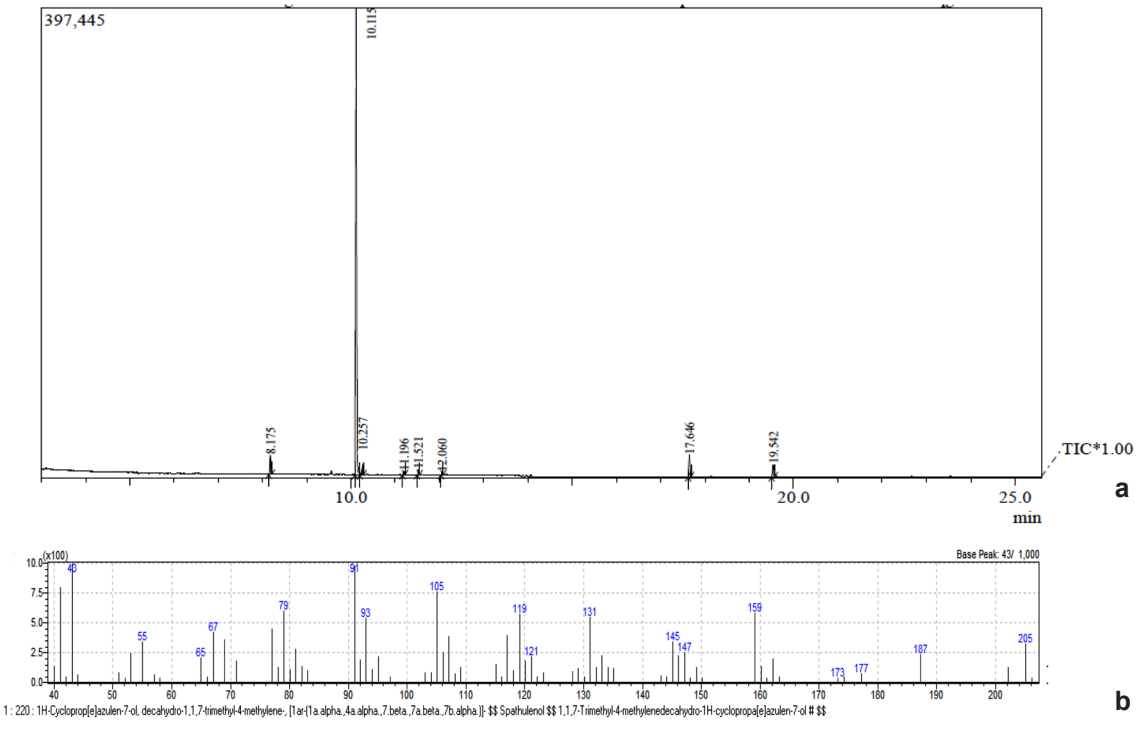

Figure 1 – a. Chromatogram of the SF5 obtained by gas chromatography coupled with mass spectrometer (GC-MS). b. Mass spectrum of the major peak at t_R 10.115 min, spathulenol.

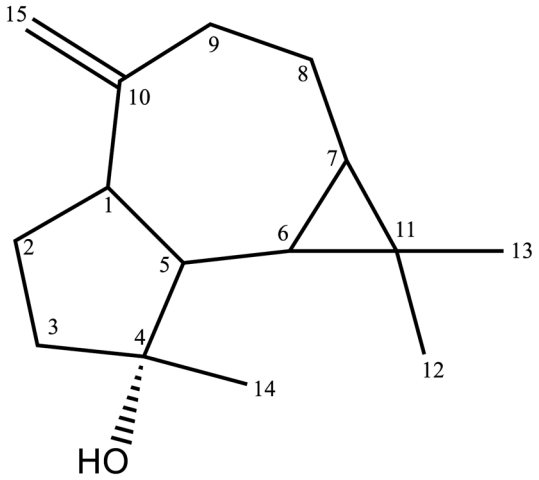

Figure 2 – Spathulenol (1).

by 52.5 ± 1.3% at 20 µg/mL), the *n*-hexane fraction and SF4 presented higher inhibitory activity on NO production and the *n*-hexane fraction was two times more active. According to the chemical profile, the major identified constituent of SF4 is an aromadendrane-type sesquiterpenoid (a spathulenol analogous), and it probably contributes to SF4 activity. Twenty-seven (27) azulenes have been reported to inhibit NO production in LPS-stimulated macrophages RAW 264.7 (Hashiba *et al*. 2004). The different compounds present in the SF2 and SF4 subfractions, with emphasis on the aromadendrane-type sesquiterpenoids as spathulenol, are associated with the inhibitory activity on NO production observed for the *n*-hexane fraction. Spathulenol showed anti-inflammatory activity in mice ear edema produced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Aguilar-Guadarrama & Rios 2004).

Thus, combining the results obtained herein with our previously reported data, it is possible to suggest that the inhibitory activity observed for the crude extract of *O. notata* on NO production might be related to an additive or synergistic effect between compounds present in *n*-hexane fraction, such as spathulenol, with other compounds (isoquercitrin and afzelin) present in ethyl acetate fraction (Costa *et al*. 2015).

Tumor necrosis factor-α (TNF-α) is a cytokine that exhibits potent pro-inflammatory capacity, mainly for its effects on diverse cells including immune cells which lead to the expression of a cascade of downstream chemical mediators. Although TNF-α is involved in various protective physiological functions and antimicrobial immunity, excessive production of TNF-α is strongly associated with fever, wasting, and tissue injury (Sedger & Mc Dermott 2014).

As seen in Fig. 3b, the *n*-hexane fraction strongly inhibited TNF-α production by LPS-stimulated macrophages RAW 264.7, 95.3 ± 0.5% at 100 µg/mL and 44.8 ± 1.7% at 20 µg/mL, with IC_{50} 15.9 ± 1.0 µg/mL. A greater inhibitory effect on TNF-α production was only seen for subfraction SF4, IC_{50} 82.1 ± 2.1 µg/mL. SF2, SF7 and SF15 subfractions presented higher IC_{50} values exhibiting a poor capacity of inhibiting TNF-α production, as also observed for spathulenol (Fig. 3b; Tab. 4). The obtained results showed that the samples were more potent inhibitors of NO than TNF-α and that the SF4 subfraction partially contributes to the inhibitory capacity of the *n*-hexane fraction.

Anti-inflammatory activity has been reported for some species of the *Ocotea* genus. *Ocotea quixos* essential oil and isolated *trans*-cinnamaldehyde were reported to reduce LPS-induced NO production and COX-2 expression on J774 macrophages and *in vivo* anti-inflammatory effects in a carrageenan-induced rat paw edema model (Ballabeni *et al.* 2010). It was also reported anti-inflammatory activity for *n*-hexane extracts of *O. bullata* on COX expression and, it was showed that *n*-hexane extracts from *O. bullata* leaves and bark inhibit *in vitro* COX-1 and 5-lipoxygenase activity (Zschocke *et al*. 2000; Madubanya *et al*. 2005). Sesquiterpene spathulenol isolated from *Salvia mirzayanii* (Lamiaceae) methanol extract showed immunomodulatory capacity of inhibiting lymphocyte proliferation (Ziaei *et al*. 2011).

The viability of the samples treated by macrophages was measured by the ability of the macrophages to metabolize MTT to formazan in order to exclude the possibility that the inhibitory effects of the studied samples on macrophages were due to their cytotoxicity. The tested samples only showed cytotoxic effect at the highest concentration (500 µg/mL), with an exception for SF7 subfraction which exhibited elevated cytotoxicity levels at 100 µg/mL (IC_{50} 16.9 ± 1.8 µg/mL). Therefore, inhibitory activities described for tested samples were generally not affected by their cytotoxicity to macrophages (Fig. 3c; Tab. 4).

Infectious diseases, including tuberculosis, are highly involved with the inflammatory response. The production of chemical mediators such as NO and TNF-α is generally essential for the immune response of mycobacteria-infected

macrophages. Nonetheless, the exacerbated inflammatory response in severe forms of TB contributes to immunopathology, requiring the use of anti-inflammatory adjunct therapy combined with the use of anti-mycobacterial drugs to prevent injury tissue and mortality (Zumla *et al*. 2014).

As can be seen in Fig. 4, the *n*-hexane fraction was notably potent against Mbv BCG. Its ability to inhibit mycobacterial growth at 100 µg/mL was 83.6 ± 1.9%, and the growth inhibition even at 20 µg/mL was of 68.1 ± 1.4 %, showing the lowest MIC_{50} values (MIC_{50} 6.6 ± 0.8 µg/mL) (Tab. 5).

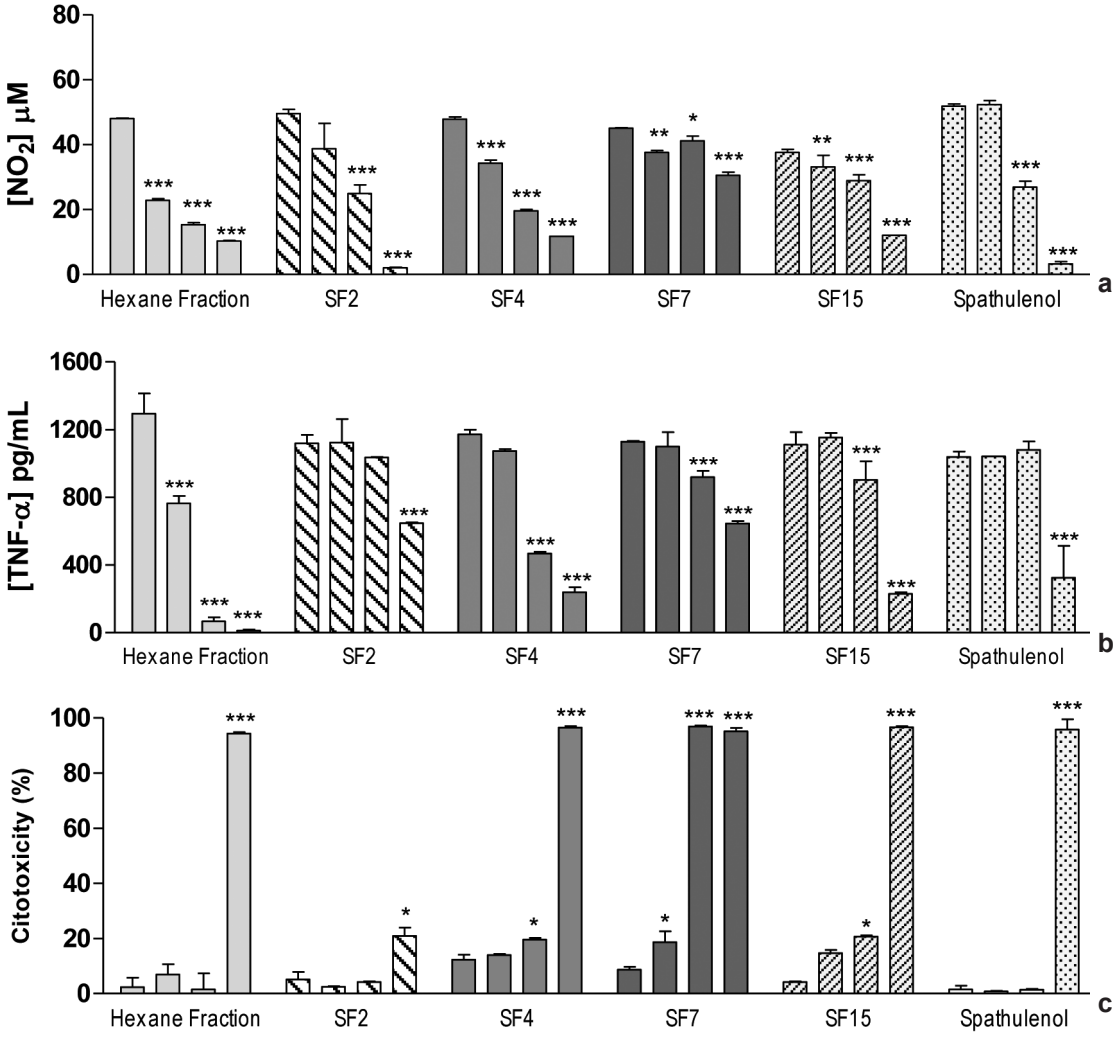

Figure 3 – a-c. Effect of *n*-hexane fraction, subfractions and spathulenol from *Ocotea notata* on NO and TNF-α production by LPS-stimulated RAW 264.7 macrophages and evaluation of cytotoxicity by MTT test. RAW 264.7 macrophages were treated with LPS (1 µg/mL) in the presence of samples (4, 20, 100 and 500 µg/mL) – a. NO production as concentration of nitrite. Untreated cells were used as a negative control (C-) and cells treated with LPS only were used as a positive control of macrophage stimulation (50.4 ± 0.5 µg/mL). Treatment with L-*N*MMA was used as a positive control of NO inhibition, reducing NO production by 52.5 ± 1.3 at 20 µg/mL; b. TNF-α production was measured using a L929 fibroblast bioassay. As a negative control it was used macrophages not treated and not LPS stimulated and as positive control macrophages stimulated with 1 mg/mL LPS and not treated (1395.6 ± 1.2 µg/mL); c. The cytotoxicity was evaluated by mitochondrial reduction of MTT to formazan and toxicity percentage was calculated in relation to the negative control (untreated macrophages, 2.2 ± 0.4%; O. D. 1.596) and to the positive control (stimulated macrophages culture treated with 1% (v/v) Triton X-100 (100.1 ± 3.6%; O.D. 0.128). * = $p < 0.05$, ** = $p < 0.01$; *** = $p < 0.001$ in relation to untreated group.

Table 4 – Minimum inhibitory concentration of *n*-hexane fraction, sub-fractions, and spathulenol from *Ocotea notata* on the production of NO and TNF-α by LPS-stimulated RAW 264.7 macrophages, and evaluation of cytotoxicity by MTT test.

Samples	IC_{50} (µg/mL)		
	NO	MTT	TNF-α
n-Hexane Fraction	8.3 ± 0.9	> 500	15.9 ± 1.0
SF2	37.7 ± 1.1	> 500	> 500
SF4	15.6 ± 1.1	> 500	82.1 ± 2.1
SF7	349.2 ± 1.3	16.9 ± 1.8	492.1 ± 1.4
SF15	241.7 ± 0.7	396.8 ± 1.4	> 500
Spathulenol	45.6 ± 2.0	> 500	392.1 ± 0.6

In comparison with our previous results (Costa *et al*. 2015), the *n*-hexane fraction showed a similar inhibitory activity profile to that seen for the crude extract of *O. notata*, and more potent than the ethyl acetate fraction on Mbv BCG growth. The SF2 and SF4 subfractions were very active, inhibiting 76.0 ± 1.1% and 69.7 ± 1.7% of Mbv BCG growth at 100 µg/mL, and 54.9 ± 0.5% and 40.6 ± 0.3% at 20 µg/mL, respectively (MIC_{50} 8.2 ± 1.0 µg/mL and MIC_{50} 12.8 ± 1.1 µg/mL*)*. The inhibitory potential of the SF7 and SF15 subfractions were low compared to others (higher IC_{50} values), while SF7 showed no selectivity for anti-mycobacterial activity, being cytotoxic to macrophages at 100 and 500 µg/mL. Spathulenol inhibited 55.4 ± 0.4% at 100 µg/mL, although its inhibitory activity was not maintained in lower concentrations, MIC_{50} 25.2 ± 1.4 µg/mL (114.3 ± 6.3 µM) (Fig. 4; Tab. 5).

We additionally evaluated the effects of the samples against the laboratory Mtb strain H37Rv and the highly virulent Mtb clinical isolate, strain M299, belonging to the modern *M. tuberculosis* Beijing sublineage. The Mtb strains were generally more resistant to anti-mycobacterial agents, as well as rifampicin, mainly against Mtb M299, since it required a ten-fold concentration of rifampicin compared to the H37Rv strain (Ventura *et al.* 2015).

The *n*-hexane fraction at 100 µg/mL substantially reduced mycobacterial growth, about

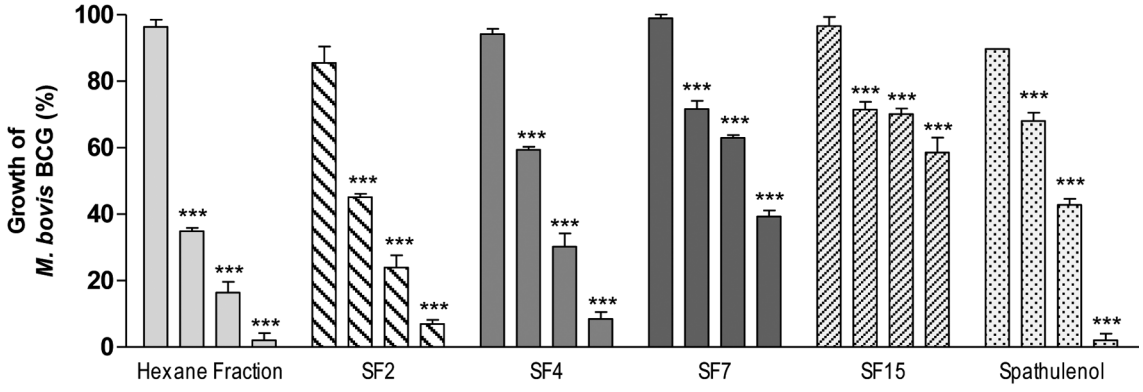

Figure 4 – Effect of *n*-hexane fraction, subfractions and spathulenol from *Ocotea notata* on growth of *Mycobacterium bovis* BCG. *M. bovis* BCG suspensions were treated with samples at concentrations of 4, 20, 100 and 500 µg/mL or rifampicin. Culture medium without bacteria was used as a positive control and culture medium with bacteria and rifampin treated-bacterial culture at concentrations of 0.001-0.03 µg/mL as negative control for *M. bovis* BCG (MIC_{50} 0.01 ± 0.3 µg/mL). ∗ = $p < 0.05$; ∗∗ = $p < 0.01$; ∗∗∗ = $p < 0.001$ in relation to untreated group.

Table 5 – Minimum inhibitory concentration of *n*-hexane fraction, sub-fractions, and spathulenol from *Ocotea notata* on the growth of *Mycobaterium* strains.

Samples	MIC_{50} (µg/mL)		
	Mbv BCG	**Mtb H37Rv**	**Mtb M299**
n-Hexane Fraction	6.6 ± 0.8	30.6 ± 1.1	35.6 ± 0.7
SF2	8.2 ± 1.0	> 500	> 500
SF4	12.8 ± 1.1	44.7 ± 0.8	37.8 ± 1.3
SF7	319.2 ± 2.3	> 500	> 500
SF15	> 500	> 500	> 500
Spathulenol	25.2 ± 1.4	36.9 ± 1.5	42.1 ± 0.5

50–55% for both Mtb strains, with MIC_{50} 30.6 ± 1.1 µg/mL (Mtb H37Rv) and 35.6 ± 0.7 µg/mL (Mtb M299) (Fig. 5; Tab. 5). Among subfractions, only the SF4 subfraction exhibited selective activity against *Mycobaterium* and reduced Mtb H37Rv and M299 growth by about 45–50% at 100 µg/mL (MIC_{50} 44.7 ± 0.8 µg/mL). Spathulenol (SF5) showed a similar activity profile against both Mtb strains, inhibiting almost 60 % of Mtb H37Rv growth (MIC_{50} 36.9 ± 1.5 µg/mL; 167.5 ± 6.8 µM) and Mtb M299 (MIC_{50} 42.1 ± 0.5 µg/mL; 191.0 ± 2.2 µM) (Fig. 5; Tab. 5).

This may suggest that the SF4 subfraction and the spathulenol are related to the *n*-hexane fraction inhibitory potential on *Mycobacterium* growth.

Previous reports have demonstrated the antimicrobial activity of the *Ocotea* species. The essential oil of *Ocotea bofo* obtained by steam distillation exhibited antimicrobial activity against yeasts. Antimicrobial activity has also been reported for *Ocotea quixos* essential oil, as methanol extract of *Ocotea odorifera* reported MIC of 180 µg/ml for the organic extract of *Ocotea* sp. against *Staphylococcus aureus* and *Enterococcus faecalis* (Yamaguchi *et al*. 2011; Suffredini *et al*. 2006).

Previous studies have attributed the antimicrobial activity of spathulenol observed in *Ocotea* extracts and fractions. Spathulenol is one of the major constituents found in *Ocotea nectandrofilia*, and partially inhibited *Cladosporium sphaerospermum* and *C. cladosporioides* growth, and presented moderate activity against *Aspergillus niger* and *Candida albicans* (Raggi 2008).

The abundant compounds found in *Espeletia nana* essential oil: α-pinene (38.1%), *β*-pinene (17.2%), myrcene (15.0%), spathulenol (4.2%), bicyclogermacrene (4.0%), *α*-zingiberene (4.0%) and himachalene (3.7%) presented antibacterial activity (MIC values were determined for *S. aureus* ATCC 25923 as 200 µg/mL and *E. faecalis* ATCC 29212 as 600 µg/mL) (Peña *et al*. 2012).

Previously, spathulenol was reported with moderate active against *M. tuberculosis* H37Rv strain (MIC 231.9 µg/mL) (Nascimento *et al.* 2018). However, recently, spathulenol, isolated from *Azorella compacta* (Apiaceae), was evaluated due to its anti-mycobacterial activity, against *M. tuberculosis* H37Rv strain and showed a MIC of 12.50 µg/mL (Dzul-Beh *et al*. 2019). It is important to mention that these two works used a different *M. tuberculosis* inoculum, 1.5×10^7 (Nascimento *et al.* 2018) and 6×10^6 (Dzul-Beh *et al.* 2019) CFU/mL, and this fact was used in the last work as a possible justification for MIC difference. In the present work, spathulenol MIC_{50} was determined as 36.9 ± 1.5 µg/mL, and *M. tuberculosis* inoculum used was 1 x 10^7 CFU/mL. Certainly, the *M. tuberculosis* inoculum seems to be important when comparing MIC values between different experiments and methodologies. Although the MIC difference, spathulenol stood out as a promising anti-mycobacterial compound and as a candidate for further studies.

In addition, a spathulenol analogous is one of the major constituents of SF4, and the antibacterial potential of aromadendrane-type sesquiterpenes has been partially investigated in the literature. Aromadendrane-type sesquiterpenes showed antibacterial activity against *Streptococcus pyogenes* methicillin, as well as sensitive and resistant strains of *Staphylococcus aureus* (Naz *et al*. 2015). Furthermore, viridiflorol, one

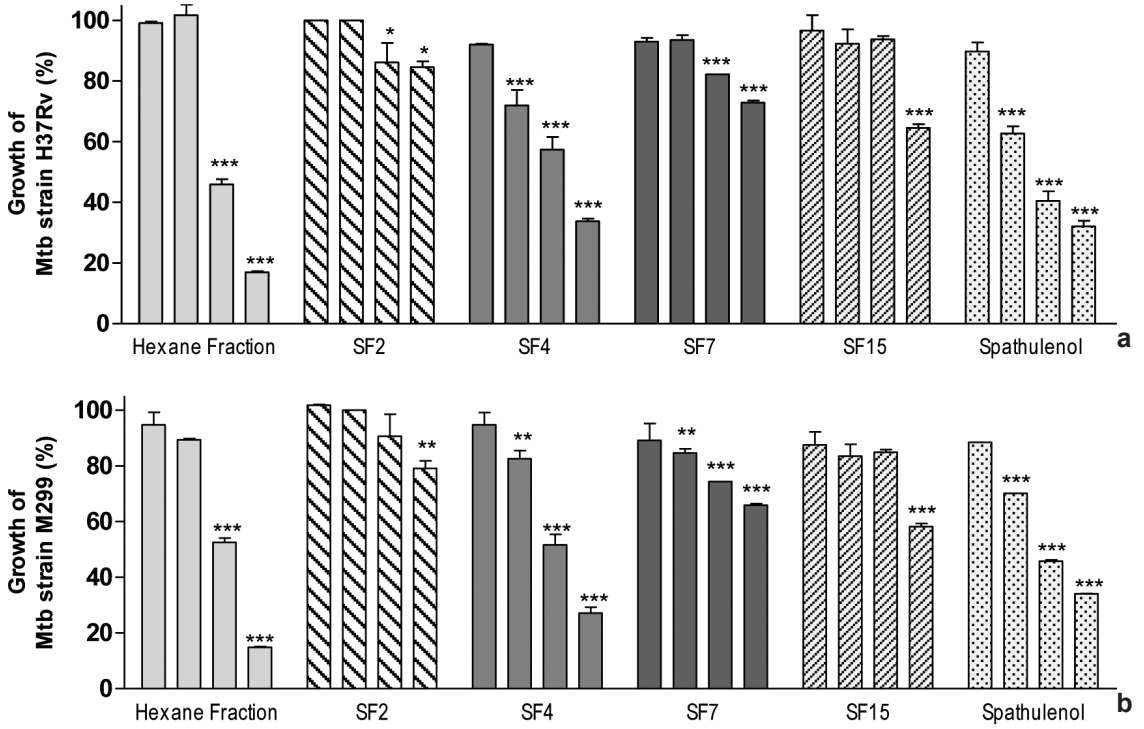

Figure 5 – Effect of *n*-hexane fraction, subfractions and spathulenol from *Ocotea notata* on growth of *Mycobacterial tuberculosis* H37Rv and clinical isolate M299. Bacterial suspensions were treated with samples at concentrations of 4, 20, 100 and 500 µg/mL for *M. tuberculosis* H37Rv (a.) and hypervirulent *M. tuberculosis* M299 (b.) or rifampicin. Culture medium without bacteria was used as a positive control and culture medium with bacteria and rifampin treated-bacterial culture (at 0.00032 to 1 µg/mL for Mtb H37Rv and at 0.008 to 10 µg/mL for clinical Mtb isolate M299) as negative control. ∗ = $p < 0.05$; ∗∗ = $p < 0.01$; ∗∗∗ = $p < 0.001$ in relation to untreated group.

aromadendrane-type sesquiterpene, has shown anti-mycobacterial activity against Mtb H37Rv with MIC 190 µg/mL (Trevizan *et al*. 2016).

In conclusion, the isolation and identification of the spathulenol sesquiterpene from *O. notata* leaves was reported for the first time in the present study, besides its anti-mycobacterial and immunomodulatory activities. It is interesting to highlight the capacity of spathulenol in inhibiting *M. tuberculosis* M299 growth, a resistant strain belonging to the Beijing family. This sesquiterpene notably contributed to the hexane fraction activity and did not show cytotoxicity when assessed on RAW 264.7 macrophages. In sum, this *in vitro* study importantly contributes to identify *O. notata* aromadendrane-type sesquiterpenes as promising anti-TB agents, which present interesting anti-mycobacterial activity combined with an immunomodulatory profile. These results show their eligibility for further *in vivo* studies in murine models of severe pulmonary tuberculosis, as recently established by our group (Almeida *et al*. 2017).

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