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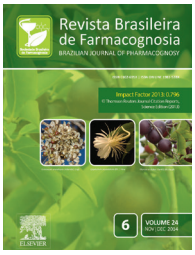

Original article

Nitric oxide production, inhibitory, antioxidant and antimycobacterial activities of the fruits extract and flavonoid content of *Schinus terebinthifolius*

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ABSTRACT

The extract of the fruits from *Schinus terebinthifolius* Raddi, Anacardiaceae, was obtained by exhaustive extraction with methanol. Its fractions and isolated compounds were collected by fractionation with RP-2 column chromatography. The crude extract, the flavonoid fraction and the isolated compound identified as apigenin (**1**), were investigated regarding its inhibitory action of nitric oxide production by LPS-stimulated macrophages, antioxidant activity by DPPH and the antimycobacterial activity against *Mycobacterium bovis* BCG. The samples exhibited a significant inhibitory effect on the nitric oxide production (*e.g.*, **1**, IC_{50} 19.23 ± 1.64 μg/ml) and also showed antioxidant activity. In addition, *S. terebinthifolius* samples inhibited the mycobacterial growth (*e.g.*, **1**, IC_{50} 14.53 ± 1.25 μg/ml). The necessary concentration to produce 50% of the maximum response (IC_{50}) of these activities did not elicit a significant cytotoxic effect when compared with the positive control (100% of lysis). The antioxidant and nitric oxide inhibition activity displayed by *S. terebinthifolius* corroborates its ethnopharmacological use of this specie as an anti-inflammatory. In addition, our results suggest that the flavonoids of *S. terebinthifolius* are responsible for the activities found. We, describe for the first time the activity against *Mycobacterium bovis* BCG and the inhibition of nitric oxide production for *S. terebinthifolius*.

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Introduction

The species *Schinus terebinthifolius* Raddi, Anacardiaceae, popularly known as pepper tree, is a native species of the Brazilian flora (Carvalho et al., 2006). Its bark, leaves and fruits have medicinal properties as febrifuge, antioxidant and anti-inflammatory (Degáspari et al., 2005; Ceruks et al., 2007).

Inflammation is a protective mechanism mediated by various chemical factors, and is comprised by complex sequential changes in order to eliminate the initial cause (Iwasaki and Medzhitov, 2010). Many diseases are followed by acute and/or chronic inflammatory processes with a high production of chemical mediators, such as atherosclerosis, Alzheimer's disease, cancer, asthma and infections, like tuberculosis (Gaestel et al., 2009). Currently non-steroidal anti-inflammatory drugs (NSAID) are the main drugs used to treat inflammation; however, these are frequently associated with gastric and cardiovascular side effects. Thus, there is a continuous need for discovery of new and less toxic anti-inflammatory drugs (Rang et al., 2007).

The physiological NO production is extremely important to defend the body; however, its overproduction and metabolites have been implicated in the development of pathologies, such as bacterial septic shock and chronic inflammation (Wadsworth and Koop, 2001). Therefore, NO production blocking agents might be beneficial for the treatment of the inflammatory response. In addition, free radical species are also responsible for activating several pro-inflammatory transcription factors, involved with the promotion of inflammatory diseases (Reynertson et al., 2008).

Thus, the aim of this work was to investigate the ability of the *Schinus terebinthifolius* fruit extract, its fractions and apigenin (**1**) to inhibit the nitric oxide (NO) production by LPS-stimulated macrophages and its antioxidant activity, as well as evaluating its cytotoxic effect, contributing to justify its popular use as anti-inflammatory, and enabling the use of this species to study the reduction of exacerbated inflammatory process. Besides the anti-inflammatory activity, the antimycobacterial activity against *Mycobacterium bovis* BCG was evaluated in order to contribute to the discovery of new antituberculosis agents.

Materials and methods

General

^{13}C and ^{1}H NMR data were obtained using a Varian 400 MHz spectrometer at the LAMAR/NPPN - UFRJ (Laboratory of Multi-User Analyses by NMR) and on a Brucker 400 MHz spectrometer at the National Center for Nuclear Magnetic Resonance Jiri Jones (Department of Biochemistry UFRJ). High Performance Liquid Chromatography analyses were performed using a Shimadzu Prominence HPLC system with two LC10AT pumps, a scanning ultraviolet SPD-M10A photodiode array detector and a Rheodyne 7725i injector. The reverse-phase column used was an RP-18 (5 µm, 250 mm, 4.5 mm i.d., Macherey-Nagel). The eluent used was purified water adjusted to pH 3.2 with phosphoric acid and acetonitrile. The following acetonitrile gradients were applied: from 0% to 15%, 5 min, 15% to 20%, 5 min, 20% to 30%, 5 min, 30% to 40%, 5 min, 40% to 41%, 5 min, 41% to 42%, 5 min and 42% to 50% for 10 min, 40 min as total time of analysis. Flow elution was 1 ml min^{-1}; 20 µl of the samples were injected.

Botanical material

Fruits of pepper tree, identified as *Schinus terebinthifolius* Raddi, Anacardiaceae, were collected at Campos dos Goytacazes, Rio de Janeiro, Brazil (Latitude 21°44'S and 41°18'W; Altitude 12 m above sea level). A voucher specimen was identified and deposited at the UENF's herbarium under the code H5073.

Preparation and fractionation of methanol extract

The fruits were cleaned and dried at room temperature for one day. Then the peel from the fruits (50 g) were subjected to exhaustive extraction with methanol (10% w/v) by static maceration for 30 days and filtered twice per week. The extract was evaporated at 35°C in a water bath in the dark. The yield of the crude extract was 12.5 g. The fractionation was performed by open column chromatography using a RP-2 column (50.0 × 5.0 cm; H_2O/MeOH gradient) affording three fractions A1 (5.0 g), A2 (3.0 g) and A3 (1.0 g). The fraction A3 (0.5 g), the only one rich in flavonoids, was subject to further fractionation with RP-2 column (25.0 × 2.5 cm; H_2O/MeOH gradient) resulting in two other sub-fractions (133.7 mg and 247.2 mg, respectively). The first (133.7 mg), with the compounds of interest, was again fractionated by RP-2 column (25.0 × 2.5 cm; H_2O/MeOH gradient) resulting in the isolated compound **1** (12.0 mg) which was analyzed by NMR (^{1}H, ^{13}C, COSY, HSQC and HMBC) for structural elucidation. ^{1}H NMR (DMSO-d_6): δ (ppm) 5.90; s (H-3), 6.05; d; *J* 1.3 Hz (H-6), 6.69; d; *J* 1.3 Hz (H-8), 6.85; d; *J* 8.6 Hz (H-3', 5') and 7.20; d; *J* 8.6 Hz (H-2', 6'). ^{13}C NMR (DMSO-d_6): δ (ppm) 167.4 (C-2), 102.7 (C-3), 181.7(C-4), 161.7 (C-5), 99.7 (C-6), 162.9 (C-7), 94.7 (C-8), 157.9 (C-9), 108.0 (C-10), 122.0 (C-1'), 128.4(C-2'), 115.9(C-3'), 161.2(C-4'), 115.9(C-5') and 128.4(C-6').

Inhibition of NO production by LPS-stimulated macrophages and cytotoxicity

The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (ATCC), grown at 37°C and 5% CO_2 in DMEM/F-12 supplemented with 10% fetal calf serum. Macrophages (1×10^5 cells/well) were seeded in 96-well plates in the presence or absence of samples at different concentrations and/or LPS [1 µg/ml] (*Escherichia coli* 055:B5). After 24 h incubation, supernatants were collected and the nitrite concentration was measured as indicator of NO production, according to the Griess test (Chi et al., 2001). Positive control: macrophages stimulated with LPS and treated with L-NMMA (Sigma-Aldrich-98% purity), a nonspecific NO synthase inhibitor at 20 µg/ml. Negative control: macrophages stimulated with LPS at 1 µg/ml and untreated.

Cytotoxic effect

The LDH release (cytoplasmic enzyme lactate dehydrogenase) was determined using the culture supernatant. The LDH release, which represents an indirect indication of cytotoxicity,

was determined using a commercial kit (Raso et al., 2001; Moraes et al., 2011). The specific release was calculated as percentage of the controls: untreated macrophages and 1% Triton X-100 (Vetec Chem.) treated macrophages.

Antioxidant activity of extracts and fractions

The DPPH (1,1-diphenyl-2-picrylhydrazyl), free radical scavenging activity of the samples were determined as described below. Samples were prepared in methanol at 2, 0.2 and 0.02 mg/ml. Samples (500 µl) were added to 500 µl of DPPH stock solution at 0.1 mM, to final concentrations of 1, 0.1 and 0.01 mg/ml. The reaction was carried at room temperature. After 1 h, absorbance values were measured at 515 nm. The radical scavenging activity (% inhibition) was expressed as percentage of scavenged DPPH and it was calculated according to the following equation: % of Inhibition = $[(A_{DPPH} - A_{sample}) / A_{DPPH}] \times 100$, where A_{DPPH} is the absorbance of DPPH solution (negative control) and A_{sample} is the absorbance of the sample in presence of DPPH. As positive control it was used 2,6-di-tert-butyl-4-methylphenol (BHT) (Sigma-Aldrich, 99% purity) (Ali et al., 2009).

Antimycobacterial activity

The mycobacterial growth inhibition was evaluated using MTT assay in 96-well plate. Initially, a suspension of *Mycobacterium bovis* BCG strain Moreau was incubated with Middlebrook 7H9 medium supplemented with 0.05% Tween 80 and ADC (Albumin Dextrose Catalase). In logarithmic growth phase, 1×10^6 CFU/well were plated in a 96-well plate, and treated with the sample at three concentrations. The plate was incubated at 37°C for seven days. After this period, the MTT solution was incubated for 3 h and the lysis buffer was added (20% w/v sodium dodecyl sulfate (SDS)/50% – dimethylformamide (DMF) in distilled water - pH 4.7). The plate was incubated overnight and the reading was carried out using a spectrophotometer at 570 nm (modified from Gomez-Flores et al., 1995). As positive control, *Mycobacterium bovis* BCG treated with antibiotic rifampin (Sigma-Aldrich-95% purity) were used, at concentrations of 0.0011 and 0.03 µg/ml. As negative control, untreated *Mycobacterium bovis* BCG was used.

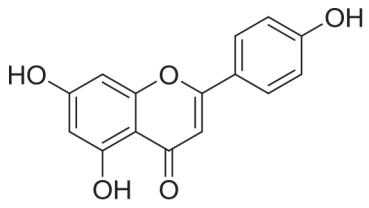
Statistical Analysis

The test was performed in triplicate and values were expressed as mean ± SD. IC_{50} values were calculated by non-linear regression.

Results and discussion

Methanolic extract from *Schinus terebinthifolius* Raddi, Anacardiaceae, fruit peels, fractions and the isolated flavonoid apigenin (**1**) were evaluated for their anti-inflammatory activity, nitric oxide production inhibitory activity and antioxidant activity *in vitro*.

Initially, the chemical profile of the methanolic crude extract and fractions were analyzed by HPLC-DAD (Fig. 1A and 1B). At 254 nm, the percentage of peak areas 1, 2 and 3 (Fig. 1B)



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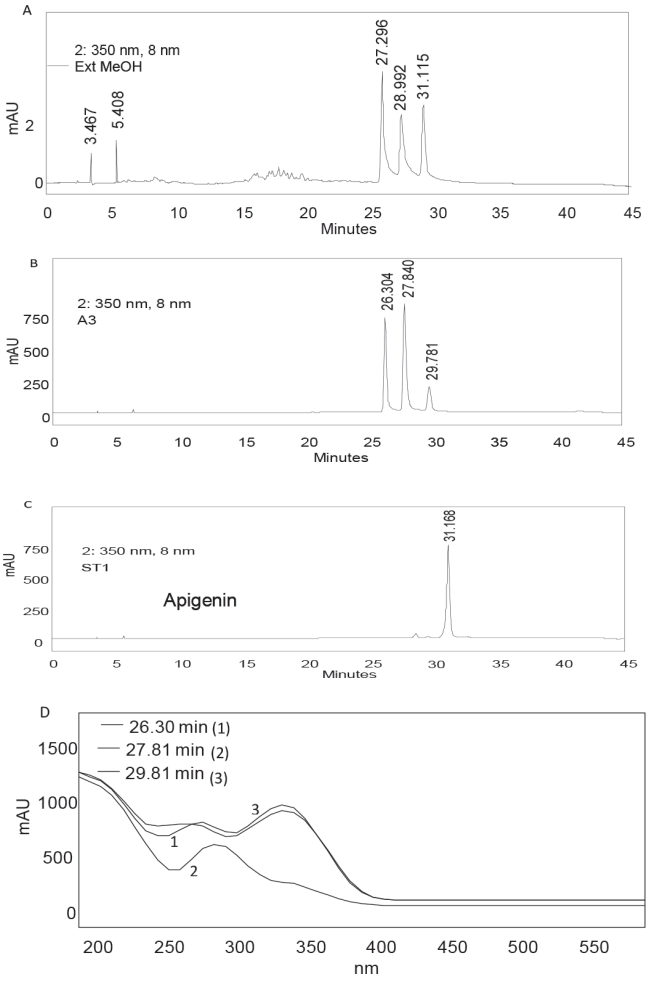

Figure 1 – High Performance Liquid Chromatography coupled with an UV Diode Array Detector (HPLC/DAD) profile of *Schinus terebinthifolius* fruits samples. Chromatograms at 254 nm of the MeOH Extract A, fraction A3 B, and apigenin C, by HPLC/DAD. Ultraviolet spectra of peaks 1, 2 and 3 of fraction A3 and their respective retention times (Tr): 26.30 min, 27.81 min and 29.81 min D.

in the retention times indicated correspond to 22.63, 15.83 and 19.46%, respectively. Both methanolic crude extract and fraction A3 showed three major peaks (retention time between 26.00 and 31.20 min) with UV spectrum typical of flavonoids (λ_{max} 250 and 350 nm) (Fig. 1D). The chromatogram of the purified compound, identified apigenin (**1**) is showed in Fig. 1C. In order to isolate the compounds responsible for the above-

mentioned activities, the extract was submitted to purification using reversed-phase open column chromatography in a RP-2 column.

Fraction A3 was purified and afforded compound **1**, identified as apigenin based on ^{1}H and ^{13}C NMR data (Agrawal, 1989; Owen et al., 2003). Although, this flavonoid was previously identified in the fruit of *Schinus terebinthifolius* by HPLC in comparison with standard apigenin (Degáspari et al., 2005), this work is the first regarding the isolation of this compound from the fruits of *Schinus terebinthifolius* and its effect in inhibiting the growth of *Mycobacterium bovis* BCG.

The anti-inflammatory potential *in vitro* was evaluated based on the ethnopharmacological use of *S. terebinthifolius* focusing on two important aspects: the inhibition of NO production by macrophages, and the ability to scavenge free radicals. The fraction A3 showed the best ability to inhibit NO production when compared with the crude extract. At the three concentrations evaluated it almost completely inhibited NO production (IC_{50} 9.25 ± 1.24 µg/ml) (Table 1). At a lower concentration (20 µg/ml), the amount of NO produced was 3.6 ± 0.40 µM (88.85 ± 1.47% of inhibition) (Fig. 2A), showing to be more active in comparison to the control treated with L-NMMA at 20 µg/ml (54.71 ± 6.21% of inhibition NO production), indicating a great inhibitory effect of NO production. Regarding the flavonoid apigenin (**1**), this compound significantly inhibited NO production (IC_{50} 19.23 ± 1.34 µg/ml) (Fig. 2B and Table 1). Apigenin activity was evaluated in lower concentrations when compared with extract and fraction because of its purity and its reduced availability.

Regarding its cytotoxicity, the extract and fraction A3 showed toxicity at 500 µg/ml near to 50%, and toxicity decreased at lower concentrations. At 100 µg/ml, for example, the cytotoxicity was reduced (Table 2) and the inhibition of NO production remained high, demonstrating that this effect is not influenced by cytotoxicity (Fig. 3A). The compound (**1**) elicited very low cytotoxicity in all concentrations tested (4, 20 and 100 µg/ml) (Table 2) supporting the data in the literature. Apigenin was proven a non-toxic and non-mutagenic flavonoid, abundant present in common fruits and vegetables such as oranges, onions, chamomile, wheat sprouts and some spices (Patel et al., 2007; Shukla and Gupta 2010).

Compounds of plant origin from different chemical classes, especially flavonoids, have been demonstrated to have anti-inflammatory activity (Coutinho et al., 2009). Apigenin, a flavone widely distributed in the plant kingdom, displays a variety of pharmacological activities, including the reduction of atopic dermatitis (Yano et al., 2009), hypotension (Loizzo et al., 2006) and anti-inflammatory properties, acting in the inhibition of inflammatory mediators such as NO and prostaglandin E2; iNOS and cyclooxygenase (COX) were also significantly inhibited *in vitro* using two different murine macrophages (RAW 264.7 and J774 A.1) induced by LPS, which suggests the apigenin mechanism of action (Raso et al., 2001).

Through the antioxidant activity carried out by the DPPH test (Table 3), it can be noted that the methanol extract and

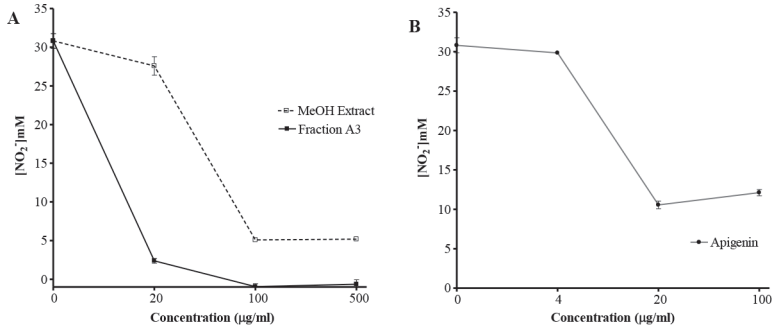

Figure 2 – Inhibitory effect of methanol extract, fraction A3 and apigenin of *Schinus terebinthifolius* fruits on nitric oxide production by LPS-stimulated RAW 264.7 macrophages. A. Methanol extract and fraction A3 at 20, 100 and 500 µg/ml; B. Apigenin at 4, 20 and 100 µg/ml. NO was indirectly quantified in culture supernatant as NO_2- by the Griess Method. Positive control - macrophages stimulated with LPS and treated with L-NMMA at 20 µg/ml (inhibiting 54.71 ± 6.21% NO production). Negative control: LPS-stimulated macrophages and untreated (inhibiting 0.0 ± 4.91% NO production). Arithmetic means ± standard deviation (n = 3).

Table 1
Expression of IC_{50} for inhibition of NO production by stimulated macrophages, specific release of LDH, antioxidant activity and growth inhibition of *M. bovis* BCG. IC_{50} is the concentration needed to produce 50% of the maximum response.

Activities	IC_{50} (µg/ml)		
	MeOH Extract	Fraction A3	Apigenin (1)
NO production inhibition	54.32 ± 1.20	9.25 ± 1.24	19.23 ± 1.34
LDH specific release - cytotoxicity	358.3 ± 1.27	463.8 ± 1.32	> 500
Antioxidant	< 10	< 10	131.9 ± 1.11
Antimycobacterial	279.5 ± 1.14	108.5 ± 1.05	14.53 ± 1.25

IC_{50} values were calculated from the results shown in Figs. 2, 3 and Tables 2, 3.

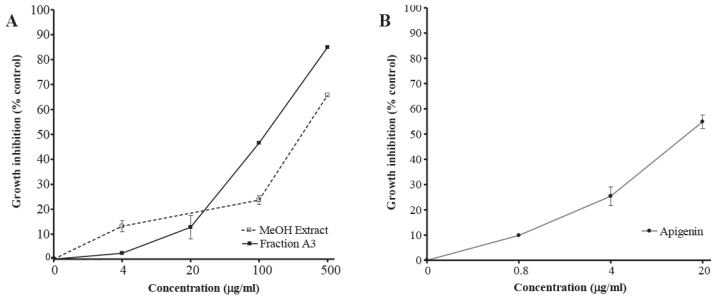

Figure 3 – Effect of methanol extract, fraction A3 and apigenin of the fruits from *Schinus terebinthifolius* on the growth of *Mycobacterium bovis* BCG evaluated by the MTT test. A. Methanol extract and fraction A3 at 4, 20, 100 and 500 µg/ml; B. Apigenin at 0.8, 4 and 20 µg/ml. Positive Control: culture medium with *M. bovis* BCG treated with antibiotic rifampin at concentrations of 0.0011 and 0.03 µg/ml (O.D. 1.144 and 0.088, respectively). Negative control: culture medium with *M. bovis* BCG untreated (O.D. 1.317). Arithmetic mean ± standard deviation (n = 3).

Table 2
Physiologic growth parameters according to treatment and vegetal organ.

Samples	LDH - specific release (% control)			
	4 µg/ml	20 µg/ml	100 µg/ml	500 µg/ml
MeOH Extract	-	26.57 ± 1.66%	35.79 ± 0.73%	52.27 ± 7.68%
Fraction A3	-	27.62 ± 2.85%	31.68 ± 3.82%	50.31 ± 1.83%
Apigenin (**1**)	3.90 ± 2.02%	3.14 ± 1.33%	6.48 ± 1.45%	-

Table 3
Percentage of antioxidant activity of methanol extract, fraction A3 and apigenin from *Schinus terebinthifolius* fruits and standard 2,6-di-tert-butyl-4-methylphenol (BHT) by DPPH assay.

Samples	Concentrations		
	10 µg/m	100 µg/m	1000 µg/m
MeOH Extract	87.6 ± 2.38%	92 ± 0.20%	95.6 ± 0.96%
Fraction A3	80.0 ± 1.2%	89.8 ± 0.9%	93.6 ± 0.9%
Apigenin (**1**)	9.0 ± 2.2%	W45.8 ± 0.9%	80.0 ± 1.2%
BHT	43.6 ± 1.5%	52.1 ± 2.2%	100.0 ± 0.9%

fraction A3 showed free radical scavenging activity at the three concentrations tested (10, 100 and 1000 µg/ml). Confirming previous literature on antioxidant activity of extracts from *Schinus terebinthifolius* (El-Massry et al., 2009).

The standard commercial BHT at the highest concentration (1000 µg/ml) showed antioxidant activity, but in comparison with the methanol extract at 10 and 100 µg/ml, the extract showed a higher antioxidant activity (IC_{50} < 10 µg/ml) (Table 1). This fact is also observed for fraction A3, demonstrating the antioxidant potential of the extract as well as the fraction. The compound **1** showed radical scavenging ability at the highest concentration (1000 µg/ml), resulting in 80% scavenging (IC_{50} 131.9 ± 1.11 µg/ml) (Table 1). For other concentrations, this compound had antioxidant activity lower than the BHT.

Literature reported that apigenin has limited antioxidant capacity (Chen et al., 1996; Galati et al., 2002; Skerget et al., 2005; Wojdylo et al., 2007). According to Ross and Kasum (2002), hydroxylated flavonoids, especially 3-OH, 5-OH, 7-OH, 4'-OH and 3'-OH, are those with greater antioxidant capacity. Another aspect that enhances the antioxidant activity is related to the presence of double bonds between carbons C-2 and C-3 (Rice-Evans et al., 1996; Harborne and Williams, 2000).

Besides the anti-inflammatory activity, our group investigated the antimicrobial activity of natural products in order to contribute to the discovery of new anti-tuberculosis agents (Moraes et al., 2011). For this purpose, *Schinus terebinthifolius* methanol extract, fraction and apigenin (**1**) were also evaluated.

Another reason to investigate antimycobacterial activity is the participation of the inflammatory process in tuberculosis. Although the production of pro-inflammatory mediators plays a protective role, essential for eliminating bacilli and granuloma formation, and maintenance, strict control of the inflammatory response is needed to prevent immunopathology in MDR and XDR tuberculosis. Specifically, since excessive and inappropriate activation of the immune system and increased production of chemical mediators leads to inflammation severity and consequently worsening of tuberculosis in these cases (Garlanda et al., 2007).

In a second part of this work, for reasons explained previously, the antimycobacterial activity of *S. terebinthifolius* extract, fraction A3 and apigenin (**1**) was studied. The extract and fraction A3 were analyzed at 4, 20, 100 and 500 µg/ml, and compound **1** at 0.8, 4 and 20 µg/ml, due to its purity. It was demonstrated that the methanol extract, fraction A3 and apigenin (**1**) inhibited the growth of *M. bovis* BCG (Fig. 3). The samples showed concentration-dependent activity. At 500 µg/ml the methanol extract was able to inhibit the growth of *M. bovis* BCG in a 65.54 ± 0.71%; and the fraction A3 in 84.70 ± 0.69% (Fig. 3A). Compound **1** was more active than fraction A3 (20 µg/ml) (Fig. 3A and B), with IC_{50} of 14.53 ± 1.25 and 108.5 ± 1.05 µg/ml, respectively (Table 1).

In a previous study with flavonoids, chalcones showed high antituberculosis activity and flavones, flavanones and flavanols moderate activity (Lin et al., 2002). In another study, using plants of northeastern Mexico for the treatment of respiratory diseases, the antimicrobial activity of 48 plant extracts were evaluated and three of these extracts exhibited activity against *M. tuberculosis*: these included the extract from fruits of *Schinus molle*, which showed acceptable activity against susceptible and resistant strains (Molina-Salinas et al., 2007).

Antimycobacterial effect was also measured for apigenin isolated from *Ficus nervosa*, Moraceae, showing a MIC of 70 µg/ml against *M. tuberculosis* H37Rv (Chen et al., 2010), in consonance with our results.

In conclusion, our results showed that *Schinus terebinthifolius* methanol extract, fraction A3, and apigenin (**1**) inhibited nitric oxide production by LPS-stimulated macrophages and presented high antioxidant activity. In addition, they showed low toxicity to RAW 264.7 macrophages. These activities together could contribute to the whole anti-inflammatory activity described for *S. terebinthifolius* and for its ethnopharmacological use. This is the first time that the activity against *Mycobacterium* was studied for *S. terebinthifolius*. Our efforts will be continuing also in view of isolating other bioactive compounds from *S. terebinthifolius*.

Author's contributions

Conceived and designed the experiments: MFM, EL, DBO. Performed the experiments: NRB, MHA, IFJCB, FMA, EPA. Analyzed the data: NBR, MHA, MFM, DBO, EL. Wrote the paper: NRB, MFM, MHA. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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