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Original Article

Biological activities and phytochemical profile of *Passiflora mucronata* from the Brazilian restinga

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ABSTRACT

In general, *Passiflora* species have been reported for their folk medicinal use as sedative and anti-inflammatory. However, *P. caerulea* has already been reported to treat pulmonary diseases. Severe pulmonary tuberculosis, generally caused by *Mycobacterium tuberculosis* strains resistant to multiple drugs, can lead to deleterious inflammation and high mortality, encouraging new approaches in drug discovery. Thus, the aim of this work was to evaluate the *Passiflora mucronata* Lam., Passifloraceae, potential for tuberculosis treatment. Specifically, related to antimycobacterial activity and anti-inflammatory related effects (based on inhibition of nitric oxide, tumor necrosis factor-alpha production and antioxidant potential), as well as the chemical profile of *P. mucronata*. High performance liquid chromatography coupled with diode-array ultraviolet and mass spectrometer analyses of crude hydroalcoholic extract and ethyl acetate fraction showed the presence of flavonoids. Ethyl acetate fraction showed to be as antioxidant as *Ginkgo biloba* standard extract with EC_{50} of 14.61 ± 1.25 μg/ml. One major flavonoid isolated from ethyl acetate fraction was characterized as isoorientin. The hexane fraction and its main isolated compound, the triterpene β-amyrin, exhibited significant growth inhibitory activity against *Mycobacterium bovis* BCG (MIC_{50} 1.61 ± 1.43 and 3.93 ± 1.05 μg/ml, respectively). In addition, *Passiflora mucronata* samples, specially hexane and dichloromethane fractions, as well as pure β-amyrin, showed a dose-related inhibition of lipopolysaccharide (LPS)-induced nitric oxide production. In conclusion, *Passiflora mucronata* presented relevant biological potential and should be considered for further studies using *in vivo* pulmonary tuberculosis model.

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Introduction

The *Passiflora* genus, Passifloraceae, comprises about 520 species (Wohlmuth et al., 2010) that are found predominantly in tropical and subtropical regions of the world (Dhawan et al., 2004). Some species, such as *Passiflora alata*, *P. edulis* and *P. incarnata* are known for their sedative properties and have relevant interest for the food and pharmaceutical industry (Dhawan et al., 2004; Zeraik et al., 2010).

Several species of *Passiflora* exhibit biological activity through production of different types of secondary metabolites, especially flavonoids, phenols and alkaloids (Dhawan et al., 2004). The aerial parts of *Passiflora caerulea* are used in folk medicine as mild antimicrobial agent in diseases like catarrh and pneumonia (Anesini and Perez, 1993). The ethanolic extract of *Passiflora incarnata* exhibits anti-inflammatory properties at a dose of 125–500 mg/kg in rats (Borrelli et al., 1996). *Passiflora edulis* leaf extract showed potential antioxidant activity (Sunitha and Devaki, 2009; Silva et al., 2013).

Passiflora mucronata Lam. was found as a climbing plant in a tropical sandy coastal plant community, a habitat locally called restinga, in the southeastern state of Rio de Janeiro, Brazil (Garbin et al., 2012). The plant communities around the Atlantic rainforest

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complex, such as restinga, differ from the core formation where they exhibit more extreme environmental conditions; drought, salinity, high temperatures and poor soil nutrition are the main limiting factors in the restinga vegetation open scrub habitat (Scarano, 2002).

To our knowledge, there have been no pharmacological studies for *P. mucronata*, but rather ecological and botanical investigations. The pollination biology of four passion flower species was studied in southeast Brazil, specifically the importance of floral nectar chemical features, pigments and odors. All species required pollinators to produce fruits and it was observed that *P. mucronata* was pollinated by bats (Varassin et al., 2001). Gas chromatography-mass spectrometry (GC–MS) analysis of dichloromethane extracts from *P. mucronata* flower fringe filaments revealed eicosene, benzyl alcohol and limonene as the main constituents (Varassin et al., 2001). *P. mucronata* has been used to treat insomnia, hemorrhoid, and as sedative and vermifuge for medicinal purposes in Quissamã, Rio de Janeiro State, a restinga area which belongs to Jurubatiba National Park (Bolosco and Valle, 2008). This National Park comprised the cities of Carapebus, Quissamã, Macaé and has a total area of 148.6 km^2 (Imbassahy et al., 2009).

Because other species of the genus *Passiflora* show antimicrobial, anti-inflammatory and antioxidant activities and in view of the lack of information on this species, it is important to investigate the secondary metabolites found in *P. mucronata* species from this region, and to question their possible biological activity. This study aimed to: (1) evaluate the activity of leaf crude hydroalcoholic extract of *P. mucronata* against *Mycobacterium*, as well as activity of its fractions and isolated compound(s); (2) verify that the extracts, fractions and isolated compounds inhibit NO and TNF-α production by the macrophages and their ability to act as an antioxidant; (3) evaluate the cytotoxicity in the macrophages, which are the *Mycobacterium* host cells; and (4) investigate the phytochemical profile of *P. mucronata* leaves.

Materials and methods

General experimental procedures

^{1}H NMR and ^{13}C NMR spectra were obtained from 400 MHz and 100 MHz, respectively, on a Bruker DRX-400 NMR spectrometer and a Varian MERCURY-VX from 400 MHz. Chemicals shifts (δ) were referenced to internal TMS standards (δ = 0, 1H) being expressed in parts per million (p.p.m.) units and the coupling constants (J) in hertz (Hz). Preparative flash chromatography was performed on silica (Siliaflash GCO; 70–230 mesh). Eluates were monitored by thin-layer chromatography (TLC) on silica 60 F254 using butanol/acetic acid/water (BAW 8:1:1), visualized under UV light and revealed with NP-PEG or using hexane/ethyl acetate (9.5:0.5) revealed with anisaldehyde/sulphuric acid. The fractions were analyzed by HPLC-DAD on Shimadzu SCL-10A with Diode Array Detector SPD-M10A with absorptions measured from 200 to 450 nm. Additionally, GC–MS analysis was performed on GCMS-QP2010 system (Shimadzu).

Plant material

Passiflora mucronata Lam., Passifloraceae, leaves were collected in February 2011 at the Restinga de Jurubatiba National Park, Quissamã, Rio de Janeiro, Brazil (22.19828° S; 41.46338° W; 10 m altitude). Voucher specimens were deposited at University Federal Rio de Janeiro Herbarium, Brazil (RFA 38758) after identification by the botanist Tatiana U. P. Konno. This research has complied with all relevant federal guidelines and institutional policies related to the botanical material for research purposes.

Extraction and isolation

Fresh leaves (50.4 g) were triturated and extracted with 500 ml of ethanol/water (85:15) at room temperature by maceration for 24 h. The solvent was renewed five times, completing the extraction after 120 h. The extract was lyophilized and then an aliquot of 6.96 g of the total dried crude hydroalcoholic extract (code PMCE) (7.42 g) was re-suspended with methanol and partitioned with hexane to obtain PMH (1.14 g). The residual methanol phase was dried and re-suspended with pure water and partitioned sequentially with dichloromethane, ethyl acetate and butanol, affording PMDM (532.9 mg), PMEA (134.6 mg), PMB (1.2465 g), respectively. The residual aqueous phase was named PMA (2.5915 g). An aliquot of 501.6 mg of PMH was chromatographed on a silica column (62.0 × 2.0 cm; hexane/ethyl acetate/methanol gradient), yielding 271 fractions. Fraction PMH-113 crystallized *via* the slow evaporation of hexane/ethyl acetate (8:2), affording compound 1 (108.6 mg) as white crystalline needles. Compound 1 was identified as triterpene β-amyrin based on MS data (GC–MS analysis), m/z 498 [$M{+}SiMe_3$]+ (trimethylsilylated β-amyrin), and NMR spectroscopy data, in accordance to literature reports (Carvalho et al., 1998). An aliquot of 54.2 mg of PMEA was chromatographed on a reverse-phase C18-bonded silica column (61.0 × 1.5 cm; distilled water/methanol gradient), yielding 48 fractions. The fractions 18–19 (85:15) and 20–22 (80:20) were combined affording compound 2 (5.3 mg). Compound 2 was identified as flavone isoorientin by NMR spectroscopy data, in accordance to literature reports (Costa et al., 2011; Wen et al., 2007).

Gas chromatography–mass spectrometry (GC–MS) analyses

The hexane fraction and isolated compound 1 were dissolved in *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, and resultant trimethylsilylated products were directly subjected to GC–MS analysis on DB column (φ 0.25 mm × 15 m, 0.25 μm film thickness, J and W Scientific) with helium carrier (flow rate 1 ml/min). Injector and interface were maintained at 270 and 230 °C, respectively. Column temperature increased from 60 to 280 °C, with a 15 °C/min temperature ramp.

Chromatographic analysis by HPLC-DAD

Crude extract from leaves and ethyl acetate were analyzed by HPLC-DAD (Shimadzu) with an RP-18 reverse-phase column (5 μm particle, 250 mm × 4.60 mm, Supelcosil, Supelco) maintained at 30 °C. The eluents were H_2O adjusted to pH 3 with H_3PO_4 (A) and acetonitrile (ACN, B). The following mobile phase gradient (v/v) was used: 0–10 min, A–B (100:0 → 82:18); 10–35 min, A–B (82:18 → 80:20); 35–40 min, A–B (80:20 → 79:21); 40–45 min, A–B (79:21 → 78:22); 45–50 min, A–B (78:22 → 0:100). The injection volume was 10 μl and the flow rate was 1.00 ml/min. HPLC analyses were performed on each sample, after dilution of 5 mg in 1 ml of ultrapure water.

Chromatographic analysis by HPLC-MS

The ethyl acetate fraction was analyzed by HPLC-MS system (Shimadzu) with an LC 20 AD pump, automatic injection SIL20AHT; Ion Trap Amazon SL Bruker (Billerica, MA), Nebulizer 70 psi; Dry gas 10 l/min; Dry Temp. 330 °C; 0.7 eV. Column Luna 100 A RP-18 reverse-phase (5 μm, 250 mm, 4.60 mm, Phenomenex). The eluents were H_2O adjusted to pH 3 by H_3PO_4 (A) and acetonitrile (ACN, B). The following mobile phase gradient (v/v) was used: 0–3 min, A–B (100:0 → 95:5); 3–10 min, A–B (95:5 → 90:10); 10–17 min, A–B (90:10 → 80:20); 18–24 min, A–B (80:20 → 75:25); 25–29 min, A–B (75:25 → 70:30); 30–39 min, A–B (70:30 → 65:35); 40–49 min, A–B

(65:35 → 55:45); 50–59 min, A–B (55:45 → 40:60); 60–69 min, A–B (40:60 → 20:80); 69–74 min, A–B (20:80–0:100); 75–76 min, A–B (0:100 → 95:5). The injection volume was 10 μl and the flow rate was 1 ml/min. HPLC analyses were performed on each sample, after dilution of 5 mg in 1 ml of ultrapure water.

Flavonoid quantification in the crude extract and its ethyl acetate fraction

The flavonoid quantification by HPLC was carried out using a ten-point calibration curve obtained using rutin (Sigma–Aldrich ≥ 94% purity) amounts ranging from 0.20 to 10.0 μg. The detector response linearity range was verified using a series of two-fold diluted rutin solutions. The relationship between peak areas (detector responses) and amount of rutin was linear over 1000–20 μg/ml (r^2 = 0.9999). To evaluate the injection integration repeatability, the rutin standard solution and samples were injected three times and the relative standard deviation values calculated.

Antimycobacterial activity

Samples were evaluated for their antimycobacterial activity using a [3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide] MTT assay to measure mycobacterial growth in a liquid medium. Initially, *Mycobacterium bovis* BCG strain Moreau (provided by Butantan Institute – Brazil) or *M. tuberculosis* H37Rv (ATCC 27294) suspension was grown in Middlebrook 7H9 medium supplemented with 0.05% Tween 80 and albumin, dextrose, catalase (ADC). During the middle logarithmic growth phase, the bacterial suspension was plated in a 96-well microplate (1×10^6 CFU/well) in the presence of each sample at four concentrations. The sealed plate was incubated at 37 °C and 5% CO_2 for 7 days for *M. bovis* BCG and 5 days for *M. tuberculosis* H37Rv. After this period, the mycobacteria were incubated for 3 h with MTT solution and lysed *via* treatment with lysis buffer (20% w/v sodium dodecyl sulfate – SDS/50% dimethylformamide – DMF in distilled water – pH 4.7) (Gomez-Flores et al., 1995). The plate was incubated overnight at 37 °C and the reading was made using a UV spectrophotometer at 570 nm. As positive control, a bacterial suspension treated with isoniazid (Sigma–Aldrich ≥99% purity), at concentrations of 0.032, 0.16, 0.8 and 4 μg/ml. As a negative control, an untreated bacterial suspension was used. Concentrations are reported in terms of μg/ml so that isolated compound could be plotted together with fractions and extract.

Determination of NO and TNF-α production from LPS-activated RAW 264.7 cells

Macrophages RAW 264.7 (ATCC, TIB-71) were cultured in Dulbecco's Modified Eagle's medium (DMEM-F12) supplemented with 10% fetal bovine serum (FBS) and gentamicin (0.2%) in the presence 5% CO_2 at 37 °C. These cells were seeded in 96-well microplates (2×10^4 cells/well) in the presence of each sample at three concentrations and stimulated with 1 μg/ml LPS (Escherichia coli 0111:B4; Sigma-Aldrich). After 24 h incubation period, culture supernatants were collected for NO and TNF-α assays. In the NO experiments, a nitric oxide inhibitor, N^G-methyl-L-arginine acetate salt (L-NMMA – Sigma–Aldrich 98% purity), was used as positive control at 20 μg/ml. As a negative control, untreated macrophages were used. Nitrite, a stable NO metabolite, was quantified by using the Griess method (Griess, 1879). The nitrite concentration was calculated from a $NaNO_2$ standard curve. The optical density was measured by spectroscopy at 570 nm. TNF-α was measured by an L929 fibroblast (ATCC, CCL-1) bioassay, based on sensitivity of L929 cells to cytotoxic effect of TNF-α. For this, the L929 cells were seeded in 96-well microplates (2.5×10^4 cells/well). After 24 h incubation, the resulting cell monolayers were treated with the macrophage culture supernatants in the presence of actinomycin D (2 μg/ml). After an additional 24 h incubation, the L929 cell viability was assayed by the MTT method (Mosmann, 1983). The cytokine concentrations were determined by using a recombinant mouse cytokine to obtain a standard curve correlating cellular viability and TNF-α concentration. Each well's optical density was measured at 570 nm employing a microplate reader (Dynatech MR5000).

Cytotoxicity

Cytotoxic effects of *P. mucronata* samples on macrophages were examined using a commercial LDH kit (Doles®). Release of lactate dehydrogenase (LDH, cytoplasmic enzyme) from RAW 264.7 cells treated with the samples was determined colorimetrically, as described previously (Muzitano et al., 2006). Briefly, the cells (2×10^4 cells/well), seeded in 96-well microplates, were treated with samples at 0.8, 4, 20 and 100 μg/ml for 24 h. Extracellular LDH concentrations were quantified in supernatant culture. Cell lysates obtained through the treatment with 1% Triton X-100 were used as a positive control. DMSO was used as solvent for the sample dilutions, and was tested in parallel as control.

Antioxidant activity assay

Passiflora mucronata extract and fractions radical scavenging activity was estimated using stable free radical 2,2-diphenyl-1-picryl-hydrazylhydrate (DPPH, Sigma). The assay was determined in 96-well microplates using a modified method (Mensor et al., 2001). The extracts and fractions were diluted to the final concentrations of 1, 5, 10, 25, 50, 100 and 200 μg/ml in methanol. *Ginkgo biloba* extract (GBE) is an important antioxidant extract and is composed mainly by terpenoids, flavonoids (Jiang et al., 2017), as well as, *P. mucronata* crude extract and ethyl acetate fraction. For an interesting comparison, the GBE was used as positive control and prepared using the same samples dilution procedures. In each well were added 125 μl of sample solution and 50 μl of DPPH solution (300 μM). The microplate was allowed to react at room temperature and absence of light. After 30 min the absorbance values were measured at 518 nm in spectrophotometer Spectra Max and converted into percentage antioxidant activity ($AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100]/Abs_{control}\}$ (Gonçalves et al., 2015). Methanol (50 μl) plus samples solution or GBE (125 μl) was used as blank. DPPH solution (50 μl) plus methanol (125 μl) was used as negative control. The EC_{50} value of each sample was calculated by non-linear regression from the plotted graph of percentage DPPH neutralization *vs.* concentration of sample.

Statistical analysis

The tests were performed in triplicate and values were expressed as mean ± standard deviation (SD). Statistical analyses were performed by one-way ANOVA, followed by Tukey's post-test. The results were considered statistically significant for $p < 0.05$. EC_{50}, IC_{50} and MIC_{50} values were calculated by non-linear regression based on the concentration-response curve of each sample by GraphPad Prism 5.

Results and discussion

P. mucronata leaf hydroalcoholic extract was partitioned with hexane (PMH), dichloromethane (PMDM), ethyl acetate (PMEA) and butanol (PMB), sequentially. From the PMH, chromatographic purification afforded compound 1, elucidated by MS and NMR as the triterpene β-amyrin. GC–MS analysis of this hexane fraction showed β-amyrin as its major component, with relative area of

37.35%. From the PMEA, the flavonoid isoorientin (luteolin 6-*C*-β-D-glucopyranoside), compound 2, was isolated and characterized by NMR.

In view of new perspectives for research of tuberculosis (TB) treatment, the antimycobacterial activity of the samples from *P. mucronata* was investigated. The search for new promising compounds that could be used as adjuvant treatment for severe pulmonary tuberculosis has been focused by our group (Machado et al., 2014; Ventura et al., 2015a,b,c). *P. mucronata* was chosen because *Passiflora* species have been reported for their popular use to treat pulmonary diseases (Anesini and Perez, 1993) and for their anti-inflammatory potential (Borrelli et al., 1996).

Currently, tuberculosis still represents a major threat to public health in several regions of the world. *Mycobacterium tuberculosis* is the main causative agent for this infectious disease in humans. In 2015 there were an estimated 10.4 million new cases of TB, including 580.000 cases of multidrug-resistant TB (MDR-TB) and 1.4 million deaths from this disease (WHO, 2016). This data shows the importance of a constant search for new antimycobacterial agents.

Natural products play an important role in this search, as plant species have shown promising *in vitro* activity against *M. tuberculosis*, with alkaloids, terpenoids and polyphenols representing the most promising classes of secondary metabolites related to antimycobacterial bioactivity (Okunade et al., 2004; Copp and Pearce, 2007; Salomon and Schmidt, 2012). This study represents the first time that antimycobacterial activity was described for *P. mucronata* and, to our knowledge, for *Passiflora* genus.

In the initial screening, samples from *P. mucronata* were able to inhibit *M. bovis* BCG growth. This attenuated strain of *M. bovis* is non-virulent, but closely related to *M. tuberculosis* (Mahairas et al., 1996). As shown in Fig. 1a, at 100 μg/ml, *P. mucronata* crude extract (PMCE) inhibited 79.22 ± 3.48% of the *Mycobacterium* growth. PMH, PMDM, PMEA, PMB and aqueous (PMA) fractions inhibited 97.92 ± 0.56, 86.21 ± 0.0, 38.63 ± 2.40, 47.50 ± 2.40 and 47.42 ± 7.98%, respectively, at 100 μg/ml (Fig. 1b–f). PMH, the most active, showed MIC_{50} of 1.61 ± 1.43 μg/ml and dichloromethane fraction showed MIC_{50} of 8.83 ± 1.17 μg/ml. Compound 1, at 100 μg/ml showed 86.76 ± 0.21% of *Mycobacterium* growth inhibition (MIC_{50} 3.93 ± 1.05 μg/ml) (Fig. 1g). Compound 2 was not evaluated due to the low activity found to PMEA.

In the next step, the three most promising samples: PMH, PMDM and compound 1 were evaluated against a virulent strain of *M. tuberculosis* H37Rv. Although inhibition percentages proved low, the fractions and compound 1 were still able to inhibit the growth of this virulent strain (Fig. 1i–k). These results are in agreement with literature data, which reported a low activity for *M. tuberculosis* growth inhibition by β-amyrin (Martins et al., 2011). A study evaluated a mixture of lupeol and α-and β-amyrin showing promising antimicrobial activity, but confirmed low activity of β-amyrin once isolated (Higuchi et al., 2011).

Inflammation is strongly correlated with the pathogenesis of most infectious diseases, including TB. In general, for protection against mycobacteria, the production of pro-inflammatory mediators, such as NO and TNF-α, by the infected macrophages is essential. However, in the cases of severe forms of TB, such as military TB or tuberculous meningitis, additional anti-inflammatory therapy is required to prevent excessive inflammation (Garlanda et al., 2007). In addition, anti-inflammatory therapy reduces mortality in patients exhibiting hyperinflammatory phenotype that could be determined by host genetic polymorphisms, increased bacterial virulence or specific comorbid states (Critchley et al., 2013).

Such aspects could also be pre-clinical investigated during new TB drug development. *In vivo* studies done by our group using TB model in C57BL/6 mice infected with the highly virulent *M. tuberculosis* strain M299 reproduce the hyperinflammatory response of a TB-resistant immunocompetent host to highly virulent mycobacteria. This response was strongly associated with excessive recruitment of polymorphonuclear and mononuclear phagocytes and proinflammatory cytokine production in the lungs that can contribute to pulmonary necrosis (Almeida et al., 2017).

The need for such an alternative treatment for TB therefore exists, especially for the severe destructive and disseminated forms of TB frequently associated with exacerbated inflammation. Perhaps an ideal solution would be dually-active compounds which exhibit both antimycobacterial and anti-inflammatory activities. Indeed, our group has described several such compounds possessing dual activities (Machado et al., 2014; Ventura et al., 2015a,b,c). The importance of development of new drugs with dual, anti-inflammatory and antimycobacterial, activities is highlighted by emergency of increasing prevalence of multidrug resistant (MDR) TB and extensively drug-resistant (XDR) TB, where approximately one in five tuberculosis isolates worldwide are resistant to at least one major first-line (Dheda et al., 2017).

In the evaluation of *P. mucronata* immunomodulatory properties, with focus on pro-inflammatory mediators, it was verified whether crude extract, fractions and compound 1 could inhibit nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) production, induced in RAW 264.7 macrophages stimulated with lipopolysaccharide (LPS). The PMCE showed IC_{50} value of 9.43 ± 1.88 μg/ml. PMH, PMDM, PMEA, PMB and PMA showed IC_{50} value of 4.24 ± 1.90, 1.95 ± 1.93, 14.69 ± 3.04, 87.27 ± 1.58 and > 100 μg/ml, respectively. Compound 1 was active at all tested concentrations (Fig. 2), with IC_{50} < 0.8 μg/ml. This suggests that β-amyrin contributes to the activity observed for the hexane fraction from which it was originated. Isoorientin, isolated from PMEA, was reported in the literature to significantly inhibit the LPS-stimulated production of NO in RAW 264.7 cells (Luyen et al., 2014).

With respect to TNF-α production, *P. mucronata* showed poor inhibitory activity (data not shown). Only hexane and dichloromethane fractions showed IC_{50} value of 44.43 ± 1.52 and 56.52 ± 1.34 μg/ml. However, TNF-α production inhibitory effects exhibited by PMH can be associated with their cytotoxicity in the highest concentration (100 μg/ml) (Fig. 3). Previously, β-amyrin obtained from *Euphorbia hirta* was evaluated for anti-inflammatory activity and showed inhibitory effect on NO production by LPS-activated RAW 264.7 macrophages by inhibiting iNOS protein expression. However, when other inflammatory factors as PGE_2, TNF-α and IL-6 were evaluated, only a slight influence of β-amyrin on theirs levels was observed (Shih et al., 2010).

When cytotoxicity was analyzed, only the hexane fraction showed a level greater than 35% in the concentration of 100 μg/ml, but this value was not maintained at low concentrations, which also showed high inhibitory effects of NO production and *Mycobacterium* growth inhibition. The remaining samples in the smaller concentrations showed no significant cytotoxicity when compared to control 0% lysis ($p < 0.05$) (Fig. 3).

In addition, extract and fractions were evaluated for their antioxidant activity by DPPH spectrophotometric method. Scavenger activity is beneficial to immune response during an inflammatory process because it decreases oxidative stress, principally when it is associated with the inhibition of inflammatory mediator production (Aguilera et al., 2017; Oz, 2017). As could be seen in Fig. 4, PMCE was active but less than *Ginkgo biloba* extract (GBE), used as positive control, with EC_{50} of 96.05 ± 1.17 and 14.66 ± 1.09 μg/ml, respectively. PMEA (EC_{50} value 14.61 ± 1.25 μg/ml) show better scavenging activity than other fractions. This result can be justified by high concentration of phenolic compounds, like flavonoids (Bendini et al., 2006; Mensor et al., 2001). Isoorientin, major flavonoid of the PMEA fraction may be the main responsible for the antioxidant activity. Whereas

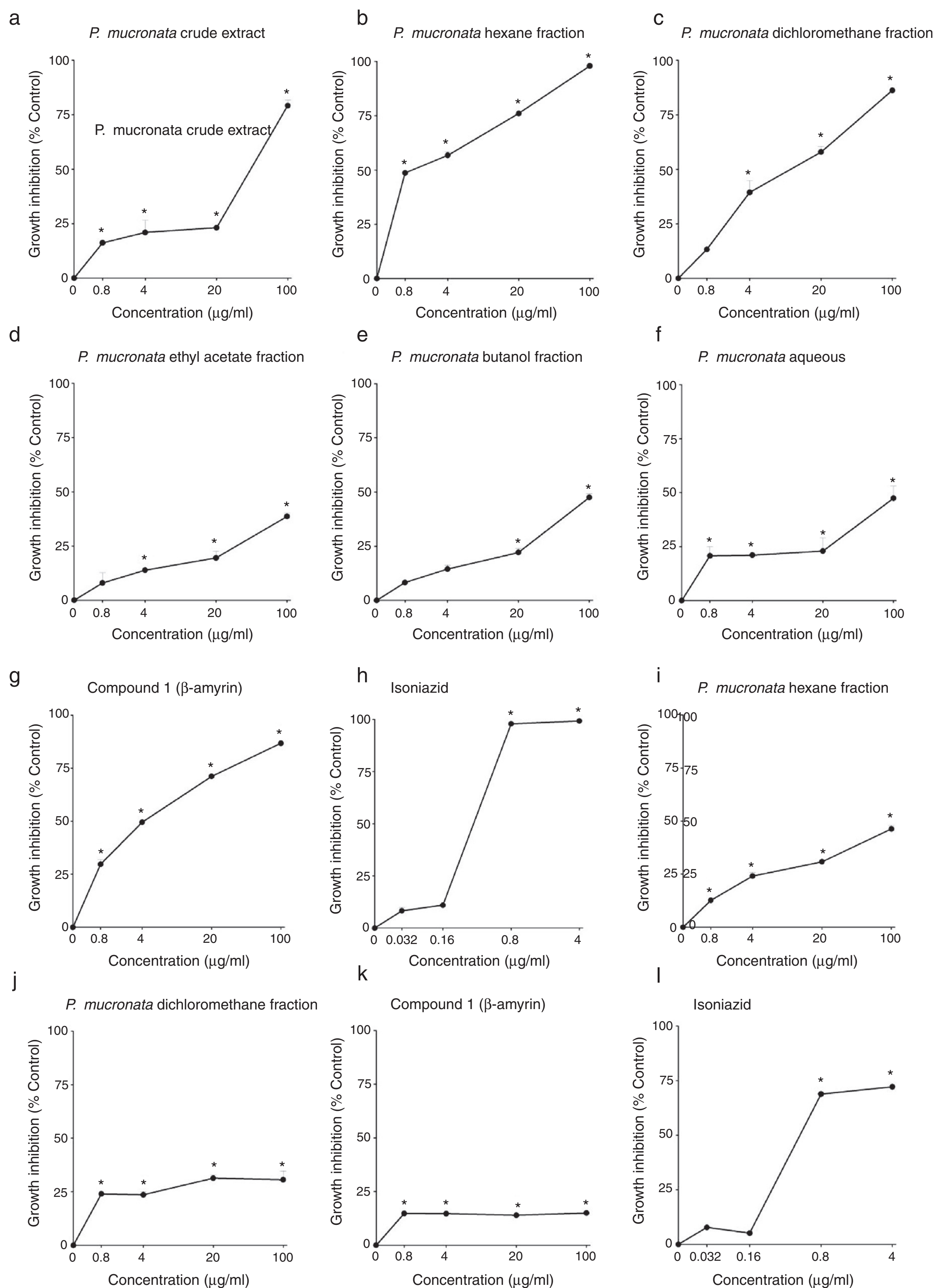

Fig. 1. Anti-mycobacterial activity of *Passiflora mucronata* extract, fractions and compound **1** at 0.8, 4, 20 and 100 μg/ml. Inhibition of the growth of *Mycobacterium bovis* BCG (A–H). PMCE (A), PMH (B), PMDM (C), PMEA (D), PMB (E), PMA (F), compound 1 (G). Positive Control – *Mycobacterium bovis* BCG treated with antibiotic isoniazid at 0.032, 0.16, 0.8 and 4 μg/ml (H). Negative Control – *Mycobacterium bovis* BCG untreated. Inhibition of *Mycobacterium tuberculosis* $H_{37}Rv$ (I–L). PMH (I), PMDM (J), compound **1** (K). Positive Control – *Mycobacterium tuberculosis* $H_{37}Rv$ treated with antibiotic isoniazid at 0.032, 0.16, 0.8 and 4 μg/ml (L). Negative Control – *Mycobacterium tuberculosis* $H_{37}Rv$ untreated. Results represented as the mean ± standard deviation of three independent experiments within triplicate. *$p < 0.05$ in relation to the untreated group.

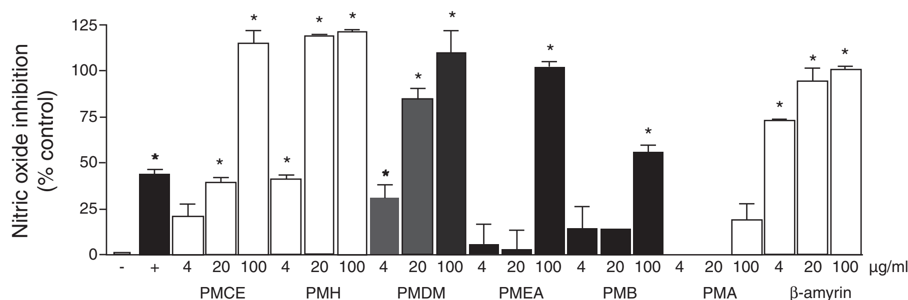

Fig. 2. Evaluation of nitric oxide (NO) production from LPS-activated RAW 264.7 macrophages treated with *Passiflora mucronata* extracts, fractions and compound 1 at 4, 20 and 100 µg/ml. Crude extract (PMCE), hexane (PMH), dichloromethane (PMDM), ethyl acetate (PMEA), butanol (PMB) and aqueous (PMA) fractions. Negative control: macrophages stimulated with 1 µg/ml LPS (0.01% inhibition and 45.77 ± 0.56 µM of NO production). Treatment with L-NMMA was used as positive control for NO inhibition, reducing 43.89 ± 4.60% of the NO production at 20 µg/ml. Results represent the mean ± standard deviation of two independent experiments within triplicate. $^*p < 0.05$ compared to negative control.

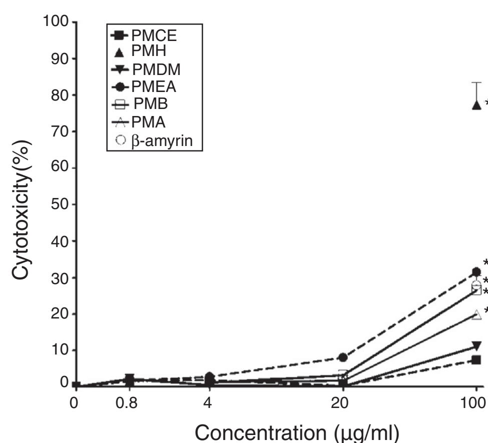

Fig. 3. Cytotoxic activity of *Passiflora mucronata* extracts, fractions and compound 1 at 0.8, 4, 20 and 100 µg/ml on RAW 264.7 macrophage cells. Cytotoxicity was measured by lactate dehydrogenase (LDH) specific release percentage. The specific release was calculated as percentage of macrophages treated with detergent Triton X-100 (1%) as positive control (Lise 100%) and macrophages non-treated as negative control (Lise 0%). Arithmetic mean ± standard deviation ($n = 3$). $^*p < 0.05$ compared to cells non-treated.

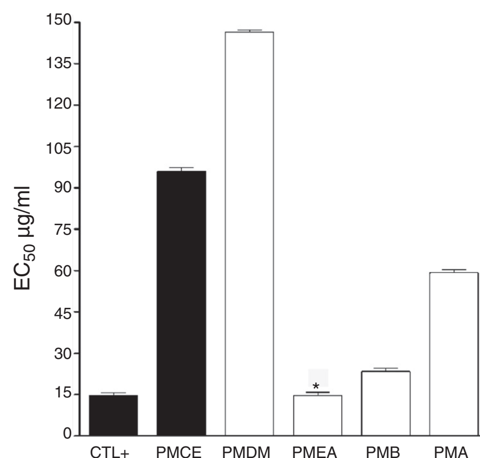

Fig. 4. Antioxidant activity of *Passiflora mucronata* extract and fractions evaluated by DPPH• assay: crude extract (PMCE), hexane (PMH), dichloromethane (PMDM), ethyl acetate (PMEA), butanol (PMB) fractions and aqueous (PMA). *Ginkgo biloba* extract was used as positive control (CTL+). The values shown represent the median effective concentration (EC_{50}) and are expressed as µg/ml. Results represent the mean ± standard deviation of three independent experiments within triplicate. *No significant difference when compared to CTL+.

isoorientin was previously reported with very high activity (EC_{50} 8.0 ± 0.2 µg/ml) and can be comparable to that of ascorbic acid in DPPH radical scavenging antioxidant activity (Sientzoff et al., 2015). Hexane fraction was not tested because its insolubility and lack of probable antioxidant characteristic compounds.

In order to better understand *P. mucronata* antioxidant activity, and also to contribute to phytochemical knowledge about this species, PMCE and PMEA were analyzed by HPLC-DAD-MS to verify their chemical profile (Fig. 5a–d). Major peaks, 14 and 15, at t_R 18.07 and 18.51 min, displayed the typical UV absorption of flavonoids with λ_{max} at 271 and 337 nm, and 269 and 349 nm, respectively (Fig. 5b). Besides major flavonoids, HPLC-DAD data allowed the identification of additional flavonoid peaks between t_R 15.77–23.32 min. The ethyl acetate fraction chromatogram showed only one major peak, number 19, at t_R 18.44 min, also characteristic of flavonoid (Fig. 5c).

HPLC-MS was used to identify the major flavonoids present in the ethyl acetate fraction (Fig. 5d, Table 1), comparing MS spectrum and elution order for isomers pairs. A number of flavonoid glycosides which have been reported in other *Passiflora* species were identified. Isochaftoside, schaftoside, isoorientin, orientin, vitexin and isovitexin are flavones considered standards used to identify different *Passiflora* species (Sakalem et al., 2012), such as *Passiflora edulis* fo. *flavicarpa, P. incanata, P. tripartite* (Sakalem et al., 2012; Zucolotto et al., 2012; Pereira et al., 2005; Abourashed et al., 2002). Flavonoid glycosides are among the compounds responsible for *Passiflora* activity in the central nervous system, as sedative-hypnotics, anxyolitic and analgesic (Sakalem et al., 2012). These identified and isolated flavonoids seem not to be involved in antimycobacterial activity, since ethyl acetate fraction presented low activity. However, they could participate in anti-TB activity of *P. mucronata* extract, using *in vivo* murine model, since they present anti-inflammatory potential, described here and in literature (Melo et al., 2005; Zucolotto et al., 2009).

Flavonoids were quantified based on an area × µg calibration curve obtained using a rutin external standard. The sum of all flavonoid peaks in chromatogram was assumed to represent *P. mucronata* extract total flavonoid content. Results are expressed as a percentage (w/w) – g/100 g of lyophilized extract. It was found that flavonoids represent 13.32% w/w of the crude extract and 14.78% w/w of the PMEA. It is important to mention *P. mucronata* extract flavonoid content was higher than others observed in plant species where flavonoids are responsible for pharmacological activity (Muzitano et al., 2011).

In conclusion, our results demonstrated that *P. mucronata* extract is a rich source of bioactive compounds. This extract and its fractions showed promising antioxidant, immunomodulatory (inhibiting the NO production) and antimycobacterial activities. In addition, β-amyrin tritepene was isolated for the first time from a *Passiflora* species and isoorientin from *P. mucronata*. In further

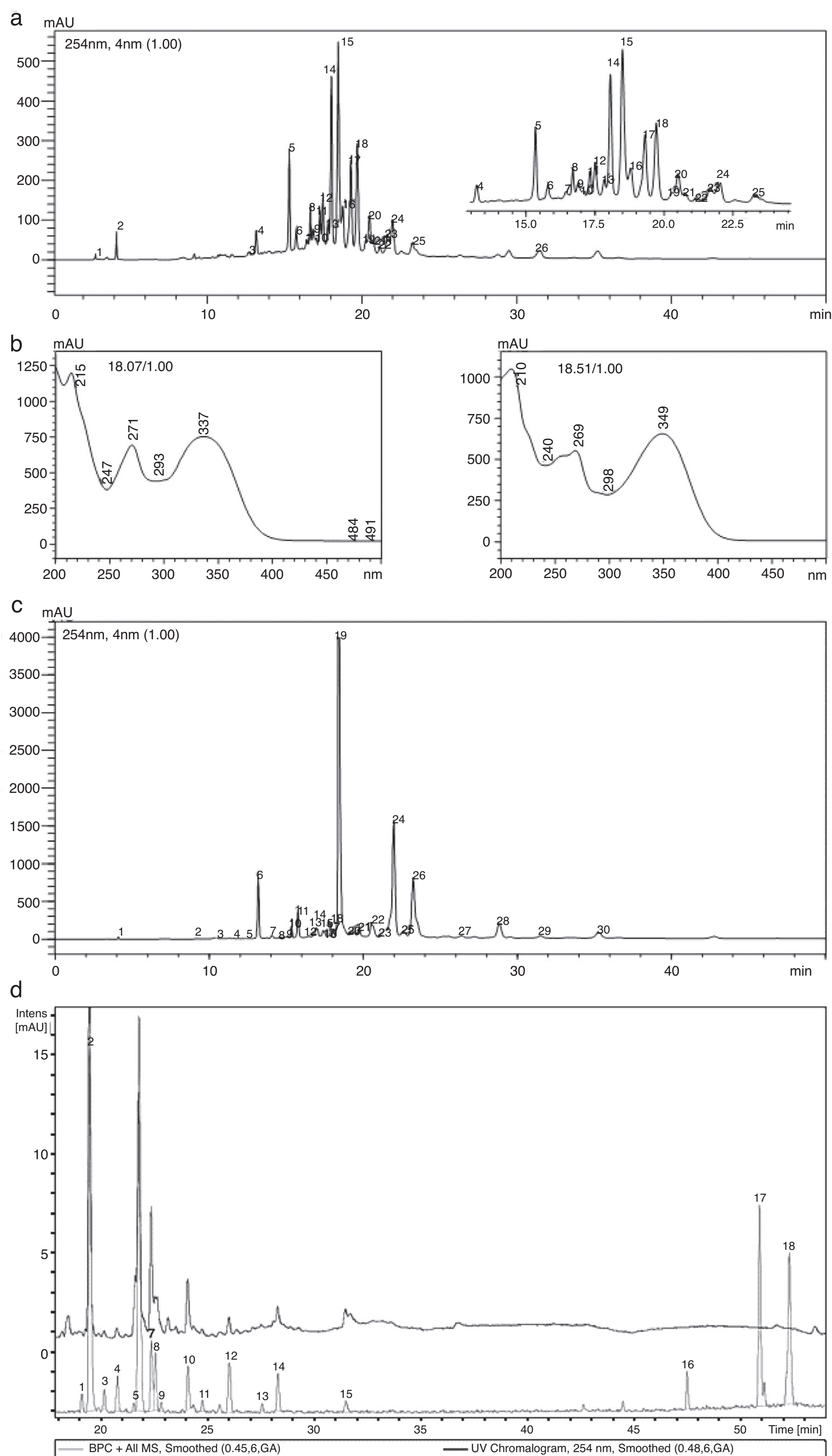

Fig. 5. *Passiflora mucronata* crude extract chromatogram at 254 nm (A). Ultraviolet spectra of crude extract chromatogram major peaks, at t_R 18.07 min (peak 14) and 18.51 min (peak 15) (B). Chromatogram of *P. mucronata* ethyl acetate fraction at 254 nm (t_R 18.44) (C). HPLC-DAD-MS chromatogram of *P. mucronata* ethyl acetate fraction, where 1-isoschaftoside; 2-isoorientin; 3-apigenin di-*C*-β-hexoside; 4-schaftoside; 5-apigenin di-*C*-β-hexoside; 6-isovitexin or vitexin; 8-methoxy luteolin-*C* hexoside; 9-orientin (D).

Table 1
The flavonoids present in ethyl acetate fraction from *Passiflora mucronata*.

Flavonoids	Retention time (min)	Molecular weight	$[M+H]^{+\cdot}$ *m/z*	References[a]
Apigenin *C*-pentoside *C*-hexoside (isoschaftoside)	19.2	564	565	[I, II]
Luteolin *C*-hexoside (isoorientin)	19.4	448	449	[I–IV]
Apigenin di-*C*-β-hexoside	20.2	594	595	[I, II, III]
Apigenin *C*-pentoside *C*-hexoside (schaftoside)	20.8	564	565	[I, III]
Apigenin di-*C*-hexoside	21.6	594	595	[II, III]
Apigenin *C*-hexoside (isovitexin or vitexin)	21.9	432	433	[I–IV]
Methoxy-luteolin-*C*-hexoside	22.6	462	463	[II]
Luteolin *C*-hexoside (orientin)	22.9	448	449	[I–IV]

[a] I, Sakalem et al. (2012); II, Zucolotto et al. (2012); III, Pereira et al. (2005); IV, Abourashed et al. (2002).

steps, it will be investigated the *in vivo* potential of *P. mucronata* active samples using a TB model, previously described (Almeida et al., 2017), useful for testing of new approaches for the treatment of severe TB, aimed at reducing the hyperinflammatory response and the prevention or reduction of pulmonary necrosis.

Authors' contributions

TUPK contributed in collecting plant sample, identification and herbarium confection. Conceived and designer the experiments: MFM, EBL, ICRL. Performed the experiments: MHA, ICVS, PFO, ARRB, FAA. Analyzed the data: MHA, ICVS, MFM, EBL, ICRL, DOG, TB, NPL, FAE. Wrote the paper: MHA, MFM, ICVS, RKD. 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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