Fitoterapia 138 (2019) 104297

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Leishmanicidal and antimicrobial activity of primin and primin-containing extracts from *Miconia willdenowii*

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ARTICLE INFO

Keywords:
Miconia willdenowii
Antimicrobial activity
Leishmanicidal activity
Leishmania amazonensis
Antifungal activity
Primin
1,4-benzoquinones

ABSTRACT

As a part of an ongoing bioprospective project, searching for potential medicinal plants from the Brazilian Atlantic Forest, *Miconia willdenowii* was selected for its potential leishmanicidal and antimicrobial activities. The crude ethanolic extract of *M. willdenowii* showed an inhibition of 99.7% of the promastigote forms of *Leishmania amazonensis* at the concentration of 80 μg/mL. Further investigation of its antimicrobial activity against pathogenic fungi and Gram positive and negative bacteria, revealed a significant antimicrobial activity. A bioguided study with its liquid-liquid partition fractions revealed the hexane fraction (Hex) as the most active against *Leishmania*, inhibiting 99.2% and 46.9% of the protozoan at concentrations of 40 and 20 μg/mL, respectively. Hex also showed significant antimicrobial activity against *Staphylococcus aureus* and *Candida krusei* with IC_{50} of 15.6 and 62.5 μg/mL, respectively. Purification of Hex led to the isolation of 2-methoxy-6-pentylbenzoquinone (**1**, also known as primin) as the active metabolite, probably responsible for the observed antimicrobial and anti-leishmania effects. Primin (**1**) disclosed leishmanicidal activity (IC_{50} = 1.25 μM), showing higher potency than the standard drug amphotericin B (IC_{50} = 5.08 μM), with additional antifungal effects against all tested fungi species. Compound **1** also showed significant activity against *S. aureus* (IC_{50} = 8.94 μM), showing a comparable potency with the reference drug chloramphenicol (IC_{50} = 6.19 μM), but with a potential cytotoxicity towards peripheral human blood mononuclear cells (CC_{50} = 255.15 μM). Here in, the antimicrobial and anti-*L. amazonensis* effects of *M. willdenowii* are reported for the first time, as well as Primin (**1**) as its probable bioactive metabolite.

1. Introduction

Leishmaniasis is a neglected tropical disease caused by protozoans of the genus *Leishmania* and Trypanosomatidae family. More than twenty species of *Leishmania* are known to be capable to infect human beings when bitten by sandflies of the genus *Phlebotomus* and *Lutzomya* [1]. *Leishmania* species are generally of zoonotic nature and transported by rodents and canids, their main hosts [2]. According to World Health Organization (WHO), leishmaniasis affects about 12 million people in 98 countries of 4 continents: Africa, Asia, America and Europe [3] and may arise in 3 different main forms: cutaneous (CL), visceral or calazar (VL) and mucocutaneous (MCL) [4]. As observed for many other infectious diseases, the therapeutics of leishmaniasis is still a challenge for medicine. The therapeutic arsenal for leishmaniasis is very restricted, with only few available drugs, some of them discovered in the early 1960′, with severe adverse effects, low efficacy and long-term

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https://doi.org/10.1016/j.fitote.2019.104297
Received 10 May 2019; Received in revised form 6 August 2019; Accepted 6 August 2019
Available online 09 August 2019

clinical regimen. The search for innovative antimicrobial drugs, with higher efficacy and less toxicity, involving diverse mechanisms of action is a current issue and deploy efforts from academic research groups and pharmaceutical industry worldwide. Most of antibacterial drugs currently available have been launched in the market in 1940s and 1960s and, in the last years, it becomes clear that these drugs are lacking from efficacy due to pathogen resistance evolution. In spite of resistance, the higher incidence of immunosuppressive diseases, such as AIDS, Crohn's disease, ulcerative colitis, and the occurrence of new or mutant pathogens have given the continuous need for research on new natural or synthetic drug candidates.

In this context, natural products are admittedly rich sources of chemical and biological diversity, being considered essential tools in the discovery and attainment of new bioactive chemical entities [5,6]. In order to investigate potential leishmanicidal plant extracts from Brazilian biodiversity, our group has focused on native plants from the remnant parts of the Southern region of Minas Gerais State. After a preliminary screening study, *Miconia willdenowii* was selected as the most promising leishmanicidal extract, inhibiting 99.7% of the promastigote forms *Leishmania amazonensis* at the concentration of 80 μg/mL.

Miconia is the biggest genus of Melastomataceae family, belonging to the Melastomatoideae subfamily and Miconieae tribe [7], with thereabout 1000 endemic species in all American tropical and subtropical regions. In Brazil, the genus *Miconia* is represented by approximately 250 native species from Atlantic Forest [7]. Biological properties of extracts and isolated substances from diverse *Miconia* species have been considerably reported in the literature, including antitumoral [8], enzyme protease inhibition [9], antimicrobial [10], antichagasic [11], antimutagenic [12], analgesic [13], leishmanicidal [14] and schistosomicidal activities [15]. Moreover, *M. myriantha* has been reported as an enzyme inhibitor of secreted aspartic proteases (SAPs) from *Candida albicans* [9]. *M. willdenowii* is a Brazilian endemic species, found in remnant areas of Atlantic forest in Rio de Janeiro, São Paulo, Minas Gerais and Paraná States [16]. Here in, we describe the bioguided study of the leishmanicidal and antimicrobial potential of the ethanolic extract of the leaves of *M. willdenowii* and its fractions, as well as the isolation of the major bioactive constituent 2-methoxy-6-pentyl-benzoquinone (**1**), also known as primin.

2. Materials and methods

2.1. General procedures

Open Column chromatography (OCC) experiments were performed by using silica gel 60 [230–400 mesh (0.200–0.360 nm), Merck] as the stationary phase, eluted with increasing polarity mixtures of hexanes/ethyl acetate, without further purification. Thin-layer chromatography (TLC) experiments employed commercial plates of silica gel PF 254 (Merck), supported on aluminum sheets. The compounds were visualized with sublimed iodine or in ultra-violet radiation chamber (λ = 254 and 366 nm). The ^{1}H and ^{13}C NMR spectra were collected on a AC-300 Bruker spectrometer (Bruker Biospin, Rheinstetten, Germany), operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. In all NMR experiments, $CDCl_3$ and tetramethylsilane (TMS) were used as solvent and internal reference, respectively. HRMS spectra were obtained for electron ionization (70 eV), while mass spectrometric analysis was performed by using a GC–MS QP-2010 (Shimadzu® Corporation, Kyoto, Japan) equipped with a RTx®-5MS (30 m × 0.25 mm i.d. × 0.25 m) capillary column (RESTEC, Bellefonte, USA). Pure helium (99.99%) with a column flow of 1.53 mL/min was used as the carrier gas. A 1 μL aliquot of the sample was injected in the split mode and analyzed under the following conditions: the injection temperature was 225 °C, and the initial temperature of the column was maintained at 80 °C for 2 min after the injection. The column temperature was then raised to 280 °C at 5 °C min^{-1} and maintained at 280 °C for 5 min. The interface and ion source temperatures were 250 and 200 °C, respectively. The purity of primin were determined by liquid chromatography and all solvents used were HPLC grade, including Milli-Q water. The HPLC system consisted of a Shimadzu modulated equipment with a two-pump gradient system incorporating Prominence LC-20AD, DGU20A 3R degasser, SIL-20A HT auto sampler, CTO-20A oven-column, diode-array UV–Vis detector (SPD-M20A), and a CBM-20A communicator. A reverse phase column (250 × 4.6 mm) Shimpack CLC-ODS (5 μm) was used, eluted with an aqueous solution of 0.1% (v/v) of acetic acid (solvent A) and acetonitrile (solvent B). The isolated primin sample was prepared at a 1 mg/mL, in a solution of water: acetonitrile (1:1), cleaned-up with hexane and filtered with a PTFE filter (13 mm i.d. × 0.45 μm). The injection volume of the sample was 10 μL and it was chromatographed using a linear gradient, with a total flow of 1 mL/min, beginning with 5% of solvent B and increasing linearly to 100% in 20 min. Afterwards, the solvent B was maintained at 100% for another 5 min, summing 25 min for the total time of analysis.

Fig. 1. Leishmanicidal activity of the subfractions **A–M** from Crude extract-Hex fraction of *M. willdenowii* at the concentrations of 20 (dark) and 40 (pale grey) μg/mL. Statistically, subfractions could be organized in 5 groups (dissimilar but similar into the same group (**1**: **F**, **G**, **H** and I; **2**: **E** and **J**; **3**: **D** and **K**; **4**: **A**, **B** and **C**; **5**: **L** and **M**, $p < .05$).

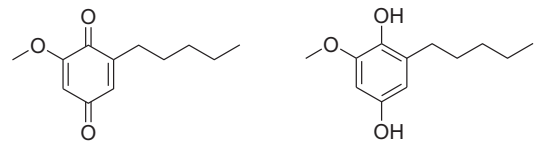

Fig. 2. Chemical structures and IC_{50} values for the bioactive metabolites primin (**1**) and miconidin (**2**), isolated from the hexane fraction of the crude ethanolic extract of *M. willdenowii*.

2.2. Plant material

The leaves of *Miconia willdenowii* Klotzsch ex Naudin (voucher number UALF2299) were collected in remnant parts of the Atlantic Forest within the Southern region of Minas Gerais (MG) State, Brazil. The species was identified by Prof. Marcelo Polo, from Federal University of Alfenas-MG, Brazil and a voucher specimen was deposited at the Herbarium of the same University.

2.3. Preparation of plant extracts, their fractions and isolation of primin from the crude extract of M. willdenowii

Leaves of *M. willdenowii* were dried in a stove with air circulation at 45 °C for 72 h. The resultant dried material (211.1 g) was ground and subjected to exhaustive extraction with ethanol for 72 h, changing the solvent (2 L) every 24 h. The alcoholic extract was then concentrated,

Table 4
Biological data for antimicrobial activity of primin (**1**).

Compound	IC μg/mL (μM)	*C. albicans* ATCC 10231	*C. tropicalis* ATCC 750	*C. krusei* ATCC 6258	*C. glabrata* ATCC 90030	*C. parapsilosis* ATCC 22019	*S. aureus* ATCC 6538	*E. coli***ATCC 25922**	*P. aeruginosa* ATCC 27853
Flu[a]	IC_{50}	0.5 (1.63)	1 (3.26)	32 (104.5)	16 (52.24)	1 (3.26)			
CHL[b]	IC_{50}						2 (6.19)	1 (3.09)	16 (49.52)
Primin	IC_{50}	15 (72.08)	15 (72.08)	**7.5 (36.04)**	**15 (72.08)**	15 (72.08)	**1.86 (8.94)**	–	–
	IC_{90}	30 (144.16)	30 (144.16)	**15 (72.08)**	30 (144.16)	30 (144.16)	**3.75 (18.02)**	–	–

[a] Flu = fluconazole.
[b] CHL = choramphenicol.

yielding the correspondent ethanolic extract (9.4 g) in 4.5% yield. The ethanolic extract was re-dissolved in a mixture of MeOH/H_2O 8:2 and the resultant hydroalcoholic mixture was submitted to a liquid-liquid partition. All phytochemical procedure, as well as the isolation of primin (**1**) and its characterization, were performed as previous reported [15].

2.4. Leishmanicidal activity against promastigote forms

The evaluation of the leishmanicidal activity of the crude extract and its fractions of the leaves of *M. willdenowii* was performed against promastigote forms of *Leishmania amazonensis* (strain MHOM/BR/71973/M2269). The parasites count was done in a Neubauer chamber, with adjust of the number parasites to a concentration of 10×10^6/mL, added to each well of a 24-well plaque, in triplicate. Then, descending concentrations of samples were added to a 6 μL volume in the wells containing the parasites. The evaluation of the crude extract of *M. willdenowii* was performed at a concentration of 80 μg/mL, whereas fractions Hex, AcOEt, hydroalcoholic and subfractions A-M were evaluated at concentrations of 20 to 40 μg/mL. For the determination of the IC_{50} values, concentrations of 40, 10, 5 and 0.1 μg/mL of miconidin and 5, 0.5, 0.1 e 0.05 μg/mL of primin were used. Despite the treated samples, growth factors were added to each plaque (LIT media and *Leishmania*), media controls (only LIT media) and controls with solvent (LIT media with parasites and the solvent used in the samples). The plaques were kept in a B.O.D incubator, at 28 °C, during 72 h. After this period, 100 μL of resazurine/well was added and after 3 h, the plaques were read in a microplate reader at 570 nm and 600 nm. The IC_{50} calculations were assisted by BioEstat 5.0 software.

2.5. Cell viability assay

Cell viability was determined using a modified MTT assay with peripheral human blood mononuclear cells (PBMC) obtained from healthy volunteers by Ficoll-Hypaque density gradient centrifugation. The cell suspension of PBMC, at a concentration of 2.4×10^6 cells/mL, was distributed in a 96-well plate, 90 μL in each well with 10 μL of test compounds at different concentrations, kept at 37 °C and 5% CO_2. After 48 h of incubation, cell morphology of the control and test wells were observed microscopically. Then, 10 μL of the dye MTT (5 mg/mL) was added and the cells were kept again for an additional 4 h period of incubation. The medium was then carefully removed and added to 100 μL of DMSO for solubilization of formazan crystals. The plates were shaken for 5 min and absorption of each sample was measured in a spectrophotometric microplate reader at 560 nm. The absorption obtained from untreated control cells was read as 100% cellular viability. Data were analyzed using linear regression to obtain values for CC_{50} (cytotoxic concentration for 50% of cells) [15,17].

2.6. Antibacterial and antifungal activity evaluation

The crude extract of *M. willdenowii*, its fractions and the pure compound **1** were evaluated *in vitro* for their antimicrobial activities through a Mueller Hinton broth microdilution method and with the methodology and interpretative criteria proposed by document M27A3 for fungi [18] and by document M07A10 for bacteria [19]. The standard pathogenic/opportunistic fungi were *Candida albicans* (ATCC 10231), *Candida krusei* (ATCC 6258), *Candida tropicalis* (ATCC 750), *Candida glabrata* (ATCC 90030); *Candida parapsilosis* (ATCC 22019). For antibacterial activity, cultures of Gram-positive *Staphylococcus aureus* (ATCC 6538), Gram-negative *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were used. The stock solutions of the tested extracts/compounds were prepared in DMSO 1% at final concentration and tested at concentrations of (μg/mL) 1000; 500; 250; 125; 62.5; 31.25; 15.625; 7.81; 3.9; 1.95 for all crude extracts and active fractions and 100; 60; 30; 15; 7.5; 3.75; 1.875; 0.938; 0.468; 0.234 for primin (**1**). Fluconazole was used as standard drug for fungistatic action at the concentrations (μg/mL) 64; 32; 16; 8; 4; 2; 1; 0.5; 0.25; 0.125; 0.0625; 0.03125, as well as chloramphenicol was used as reference drug of bacteriostatic action at concentrations of (μg/mL) 16; 8; 4; 2; 1; 0.5; 0.25; 0.12; 0.06; 0.03. The microplates were kept at 35 °C for 24 h for bacteria and 37 °C and for 24 h for fungi. Results were visualized and analyzed by spectrophotometry. The inhibitory concentration of microbial growth was determined at 50% (IC_{50}) and compared for each compound and microorganism. All tests have been performed in duplicate and the results obtained from the replicas were coincident.

3. Results and discussion

The crude ethanolic extract (CEE) of the leaves of *M. willdenowii* was prepared and screened for leishmanicidal activity, showing an inhibitory activity of 99.7% of the parasites of *L. amazonensis* in a concentration of 80 μg/mL. For such promising result, it was then further investigated in a bioguided phytochemical procedure. The CEE from the leaves of *M. willdenowii* was then extracted successively with hexanes and ethyl acetate, furnishing the corresponding hexane (Hex), ethyl acetate (AcOEt) and hydroalcoholic (Hyd) fractions, that were submitted to biological evaluation. According to our results, fraction Hex was the most active against *L. amazonensis*, inhibiting 99.2% and 46.9% of the protozoan at the concentrations of 40 and 20 μg/mL, respectively, in a concentration-dependent manner. The other two fractions showed almost the same inhibitory activity, being equivalent in the inhibition of around 42% and 16% of the protozoan activities at 40 and 20 μg/mL, respectively.

Considering the lack in the literature about the potential biological activity of *M. willdenowii*, we decided also investigate its antibacterial and antifungal properties. In the evaluation of the susceptibility profile, CEE showed significant antibacterial activity (Table 1, Supplementary Material), inhibiting 90% of growth of *S. aureus* (IC_{90} = 250 μg/mL)

and antifungal activities for *C. glabrata* (IC_{50} = 125 μg/mL) and *C. parapsilosis* (IC_{50} = 125 μg/mL). In view of these results, fractions Hex, AcOEt and Hyd were also submitted to the same antimicrobial screening experiment, with fraction Hex exhibiting the most significant antibacterial activity, especially for *S. aureus* with an IC_{50} = 15.6 μg/mL. Further investigation of antifungal effect revealed this same fraction with higher activity against *C. krusei* (IC_{50} = 62.5 μg/mL), also inhibiting *C. albicans* and *C. glabrata*, *C. tropicalis* and *C. parapsilosis* with IC_{50} values of 125, 125, 250, respectively (Table 1, Supplementary Material).

Considering that fraction Hex showed the best bioactivity profile in both leishmanicidal and antimicrobial screening assays, it was additionally submitted to a bioguided purification aiming the isolation of its active metabolite. Thus, an aliquot of fraction Hex was fractionized in an opened chromatography column (OCC), using silica gel as stationary phase, eluted successively with hexanes, ethyl acetate and methanol in increasing polarity gradient, leading to thirteen new subfractions (**A–M**, Table 2, Supplementary Material). All these fractions were tested, in parallel, against bacteria and fungi strains and promastigote forms of *L. amazonensis*.

The results for leishmanicidal activity (Fig. 1) highlighted subfractions **F**, **G**, **H** and **I** with the best inhibitory activities of 98.2, 99, 99.2 and 98.9% at a concentration of 20 μg/mL, respectively, followed by subfraction **E** that inhibited 87.5% and 65.4% of the leishmanicidal activity at 40 μg/mL and 20 μg/mL, respectively. All other subfractions did not showed significative leishmanicidal activity (lower than 40%), independently of the concentration used.

In the antimicrobial evaluation, subfractions **E–I** also showed the best inhibitory effects on bacteria and fungi strains (Table 3, Supplementary Material).

A comparative analysis by thin layer chromatography (TLC) showed that subfractions **E–J** have similar composition, leading us to group them in one single sample for additional purification. After successive purifications by OCC, two powder compounds were isolated. The combined spectroscopic analysis by IR, NMR (^{1}H and ^{13}C) and LC-MS (Supplementary Material) led to the identification of the two major constituents as 2-methoxy-6-pentyl-1,4-benzoquinone (**1**, 268 mg), a known metabolite also called as primin, and the hydroquinone miconidin (**2**, Fig. 2), the biosynthetic precursor of primin. Analytical data are in agreement with published data [15].

Further evaluation of these two metabolites against promastigote forms of L. *amazonensis* revealed primin (**1**) as a very potent leishmanicidal compound, with IC_{50} = 0.26 μg/mL (1.25 μM), showing a 4-fold higher potency than the reference drug anfotericine B (IC_{50} = 4.70 μg/mL or 5.08 μM). On the other hand, miconidin (**2**) showed to be very less active (IC_{50} = 32.3 μg/mL; 153.80 μM), suggesting that the oxidazed hydroquinone structural form is determinant for bioactivity.

The antimicrobial evaluation of primin (**1**) revealed a significant antifungal effects against all tested species, being almost 3-fold more potent than fluconazole on *C. krusei* (IC_{50} = 36.04 μM) and almost equipotent to the drug reference against *C. glabrata* (72.08 μM), Table 4). For bacteria strains, compound **1** showed significant activity only against *S. aureus* with IC_{50} of 8.94 μM, being almost equipotent to the drug reference chloramphenicol (IC_{50} = 6.19 μM).

Quinones are known as source of stable free radicals, the semiquinone radicals, as well as for their ability to irreversible complexation with nucleophilic protein amino acids, could leading to inactivation and loss of protein functionality. Due to these properties, quinones are recognized by their potential as antimicrobial agents. Considering a possible mechanism of antimicrobial activity, the most probable targets in the microbial cells are the polypeptides in cellular wall, the membrane-bound enzymes surface-exposed adhesins [5]. Other authors propose that the π-electron delocalization in the quinone structure increase its lipophilicity and, in turn, facilitate its penetration across lipid membranes, narrowing fungal and bacterial proliferation [20].

Primin (**1**) is a 1,4-benzoquinone, isolated for the first time by Bloch and Karrer from *Primula obconia* in 1927. However, only 40 years later its chemical structure has been elucidated as 2-methoxy-6-pentyl-1,4-benzoquinone [21]. More recently, primin was also identified and isolated from some species of *Miconia* (Melastomaceae), including the root extract of *M. eriodonta* [22,23], wood extract of *Miconia* sp. [24], *M. lepidota* [8] and from cultures of the endophytic fungus *Botryosphaeria mamane* PSU-M76 [25]. Among its biological properties, compound **1** exhibits allergenic effects and is responsible for contact dermatitis caused by the leaves of *Primula obconia* [26–28]. In an earlier study, we have evidenced the potential *in vitro* cytotoxicity of **1** towards PBMCs with a CC_{50} value of 255.15 μM and selective index of 7.5 [15]. However, in another study primin was reported with a moderate cytotoxicity (IC_{50} = 15.4 μM) on mammalian cells (L6) [29]. The sensitizing effect of primin and other *para*-benzoquinones has been ascribed to their probable covalent binding to protein receptors in epidermal cells involving the C(3) and C(5) sites of the quinone ring system, which could be susceptible to nucleophilic attack by the receptor [28,30]. Primin is also known by showing an important pharmacological potential due to its antitumoral [8,31], antibacterial and antiprotozoal activities, but still underexploited [29]. The first synthesis of primin was reported in 1967 and its structure is until today used as prototype and inspiration for the design of synthetic derivatives with multiple biopharmacological properties [21,32,33]. Notwithstanding the antimicrobial activity of other *Miconia* species has already been described, it has been attributed not to quinone metabolites, but to the presence of acid triterpenes [10,34] and flavonoids [35] as bioactive constituents.

In addition, Tasdemir and coauthors reported the antituberculotic and antiprotozoal activities of primin, which was described as a promising lead molecule for the treatment of African trypanosomiasis, with an IC_{50} values of 0.144 μM and 0.711 μM against *Trypanosoma brucei rhodesiense* and *L. donovani*, respectively [29]. *Leishmania* species have showed differences in the sensitivity to different compounds tested [36]. Taken together, all these results are suggestive that primin could play an important role as a lead prototype for the rational design of more potent and less toxic antiprotozoal agents [21].

4. Conclusion

In this work, we report for the first time in the literature the antimicrobial properties of the leaves extract of *M. willdenowii*, as well as evidences that the most abundant constituent primin (**1**) is the major bioactive metabolite. Our results corroborate other reports about the antibacterial activity of primin, especially against *S. aureus* [33], but also highlight, for the first time, its effect against *L. amazonensis* and antifungal potential against *C. krusei* e *C. glabrata*.

Acknowledgements

The authors are grateful to the Brazilian Agencies CNPq, FAPEMIG, PRPPG-UNIFAL-MG for financial support and fellowships. This study was also financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2019.104297.

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