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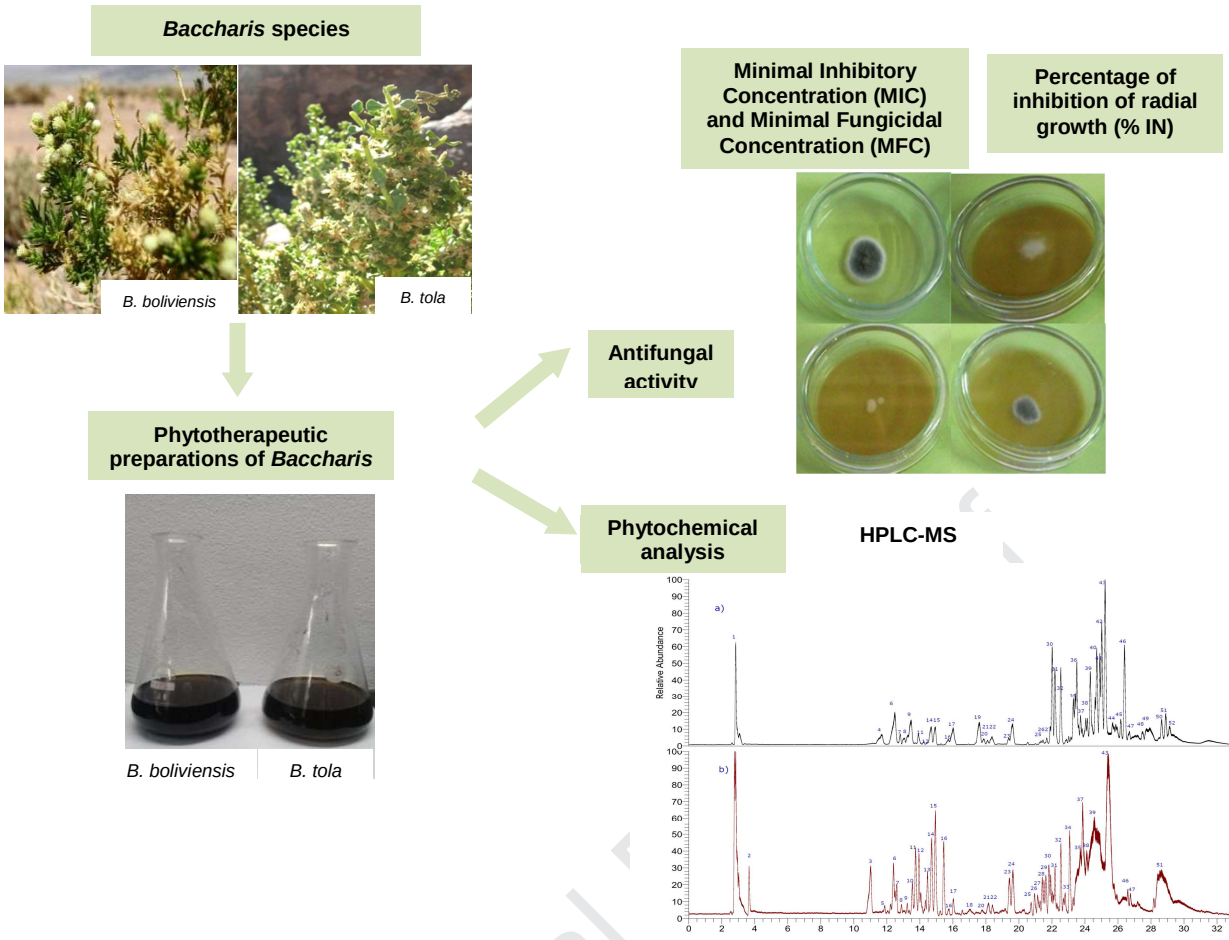
Graphical abstract



Antifungal activity of phytotherapeutic preparation of *Baccharis* species from argentine Puna against clinically relevant fungi.

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Abstract

Ethnopharmacological relevance: *B. boliviensis* and *B. tola* are used in traditional medicine in the Argentine Puna to treat skin and soft tissue infections and inflammatory processes in humans and animals.

Aim of the study: To assess the potential of phytotherapeutic preparations of *Baccharis* species as antifungal agents against clinically relevant fungi and to determine the chemical composition of the extracts.

Material and methods: Phytotherapeutic preparations of *B. boliviensis* and *B. tola* collected in Argentine Puna were evaluated as an antifungal agent against clinically relevant fungi (yeast, non-dermatophytes, and dermatophytes) isolated of patients from a local Hospital, and reference strains, using macrodilution and microdilution assays. The bioactivity was supported by UHPLC-OT-MS metabolome fingerprinting.

Results: The results revealed that the plant preparations were active against most of evaluated fungal strains; *B. boliviensis* was more active than *B. tola*. Dermatophyte fungi strains were the most sensitive isolates. The phytotherapeutic preparation showed Minimal Inhibitory Concentration (MIC) values between 25-400 µg GAE/mL and Minimum Fungicidal Concentration (MFC) values between 50 and 400 µg GAE/mL. Regarding the phytochemical analysis, total phenolic and total flavonoid contents of hydroalcoholic preparation of *B. boliviensis* were greater than those of the *B. tola* extract. Both *Baccharis* species showed similar chromatographic patterns, fifty-two compounds were identified based on UHPLC-OT-MS including several terpenoids, flavonoids and phenolic acids that have been identified in this two endemic South American *Baccharis* species for the first time. Several identified compounds present antifungal properties, the presence of these compounds support the bioactivity of the *Baccharis* extracts.

Conclusions: In this work the traditional use of both *Baccharis* species as an antimicrobial against commercial products resistant fungal strains was validate, principally against dermatophytes fungi such as *T. rubrum*, *T. mentagrophytes*, *M. canis,* and *M. gypseum*. These results indicate that the hydroalcoholic preparations could be used for the treatment of fungal infectious.

Keywords: *Baccharis tola*, *Baccharis boliviensis*, Argentine Puna, Antifungal activity, Dermatophytes.

Introduction

In recent years, there was a notable increase in the frequency and severity of fungal infections, caused not only by agents already known but by emerging pathogens, until recently considered non-pathogens or laboratory contaminants. This is justified by many reasons, the most important being the increase in patients with risk pathologies, such as cancer with induced neutropenia, transplanted patients submitted to immunosuppressive therapies or patients with HIV (Armstrong-James et al., 2014; Bongomin et al., 2017; Chapman et al., 2017; Sowmya et al., 2015). Other predisposing factors for opportunistic human mycoses are nutritional factors, and the abuse of drugs such as broad-spectrum antibiotics, corticosteroids, and immunosuppressants (Denning and Bromley, 2015; Roemer and Krysan, 2014).

The high rates of morbidity and mortality caused by fungal infections are associated with the current limited antifungal arsenal and the high toxicity of the available drugs (Scorzoni et al., 2017). On the other hand, some fungi of clinical importance present natural resistance to antifungal agents. In some cases, resistance is not innate but acquired as a result of previous antifungal therapy (Khosravi et al., 2018). For this reason, new antifungal agents are being sought. It has been demonstrated that medicinal plants are an important source of new biologically active compounds with antimicrobial activity (antibacterial and antifungal) as well as anti-inflammatory, among other properties with possible therapeutic effects (Abu-Darwish et al., 2013; Di Ciaccio et al., 2018; Mesa Arango et al., 2004; Moreno et al., 2018a, b, c; Nuño et al., 2014; Palavecino Ruiz et al., 2016; Salhi et al., 2017; Sayago et al., 2012; Torres-Carro et al., 2015, 2017; Zacchino et al., 2003; Zampini et al., 2009b, 2012).

The Andes highlands, known as Puna, extends throughout southern Peru, west of Bolivia, north of Chile and northwest of Argentina at altitudes ranging from 3300 to 5000 meters above sea level (masl). The Argentine Puna comprises part of Jujuy, Salta, Tucumán, Catamarca, La Rioja and San

Juan provinces, in an altitudinal level ranging between 2600 and 4800 masl (Figure 1). This area is characterized by scarce rainfall during the year, low atmospheric pressure and high UV radiation and extreme temperature conditions (Cabrera, 1968). The species that live in these ecosystems develop adaptation mechanisms to survive such as morphoanatomic changes as well as the production of secondary metabolites against abiotic stress. *Baccharis* species belong to the Asteraceae family, some of those that grow in the Argentine Puna, such as *B. boliviensis* and *B. tola* (Figure 2), are frequently used by local people in traditional medicine as infusion, decoction and ethanol maceration of the aerial parts to treat skin infections (wounds, burns and ulcers), as anti-inflammatory and as gastric protective agents (Abad Martinez et al., 2005; Abad and Bermejo, 2007; Calle et al., 2017; Torres-Carro et al., 2017; Villagrán et al., 2003; Zampini et al., 2009a, 2009b). Some of their medicinal properties have been validated for both *Baccharis* species: antibacterial activity against Gram-positive and Gram-negative antibiotic-resistant bacteria (Nuño et al., 2012; Zampini et al., 2009a, 2009b), antioxidant activity (Nuño et al., 2012; Zampini et al., 2008) and anti-inflammatory activity (Alberto et al., 2009; Torres-Carro et al., 2015). As well, some terpenoid and phenolic compounds were identified in *B. boliviensis* (Abad Martinez et al., 2005; Calle et al., 2012; Cazón et al., 2002; Zdero et al., 1989) and *B. tola* (Abad Martinez et al., 2005; Desmarchelier et al., 2000; Faini et al., 1982; Givovich et al., 1986; San Martín et al., 1980, Simirgiotis et al., 2016; Zampini et al., 2009a). Nevertheless, a survey of the literature revealed that there are no studies on the validation of the antifungal capacity of these plants.

In the present investigation, the activity of phytotherapeutic preparation of *B. boliviensis* and *B. tola* from Argentine Puna was studied against clinically relevant fungi (yeast, non-dermatophytes, and dermatophytes). Chemical characterization of both phytotherapeutic preparations was also included in the study.

2. Material and Methods

2.1 Plant material

Baccharis boliviensis (Wedd.) Cabrera var. *boliviensis* and *Baccharis tola* Phil. ssp. *tola* were collected during the month of February 2018 in Antofagasta de la Sierra, Catamarca, Argentina, at 3800 masl (Figure 1). The plants were identified by Dra. Ana Soledad Cuello, INBIOFIV (CONICET) and voucher specimens were included in the Herbarium of Fundación Miguel Lillo, San Miguel de Tucumán, Tucumán, Argentina (*B. boliviensis*, 607936 LIL, *B. tola*, 607934 LIL).

2.2 Phytotherapeutic preparation of Baccharis

Aerial parts of plant material were dried, grounded, and macerated in a hydroalcoholic solution (10 g dry tissue in 100 mL of 80% ethanol) for 7 days at room temperature. The extracts were passed through filter paper Whatman N°.1 to remove plant debris. The extracts were concentrated "in vacuo" at 40 °C, and then freeze-dried. The dry extracts were stored at −20 °C, until use.

2.3 Phytochemical analysis

2.3.1. Determination of total phenolic and flavonoid contents

Total phenolic compounds content was determined by the Folin–Ciocalteu method (Singleton et al., 1999). Results were expressed as µg of gallic acid equivalents (GAE) per mL (µg GAE/mL).

Total flavonoids were estimated using the method of Woisky and Salatino, (1998). Results were expressed as µg of quercetin equivalents (QE) per mL (µg QE/mL).

2.3.2. UHPLC-PDA-MS Instrument.

A Thermo Ultimate 3000 UHPLC system connected to a Thermo Q exactive focus used in this study allowed a rapid separation with photodiode (PDA) detection and high flow rates (0.8 mL per min), zero dead volume with viper technology, using effective negative electrospray ionization of the improved heated electrospray probe (HESI II), and thus getting ultrahigh resolving power performance with selectivity of a quadrupole and the orbital trap (70,000 FWHM at m/z 200), and

the outstanding diagnostic power of a high resolution collision (HCD) cell. For the analysis 10 mg of the extract were dissolved in 2 mL of methanol, filtered (PTFE filter, 200 microns) and 15 μL were injected in the instrument, with all specifications set as previously reported (Simirgiotis et al., 2016).

2.3.3. LC-MS parameters.

Liquid chromatography was performed using a C-18 column (HPLC-Acclaim, 2.5 μm, 150 mm × 4.6 mm ID, Thermo, Bremen, Germany) operated at 25 ◦C. The detection wavelengths were 330, 254, 280, and 354 nm, and photodiode array was recorded between 800-200 nm for UV characterization. Mobile phases were 1 % formic aqueous solution (A) and acetonitrile (B). The gradient program (time (min), % B) was: (0.00 min, 3% B); (5.00 min, 3% B); (10.00 min., 20% B); (15.00 min, 20% B); (20.00 min, 45% B); (25.00 min, 65% B); (35.00 min, 3% B) and 15 min for column equilibration before each injection. The flow rate was 0.8 $mL.min^{-1}$, and the injection volume was 15 μL. Standards and the resin extract dissolved in methanol were kept at 20 ◦C during storage in the autosampler. The HESI II and Orbitrap spectrometer parameters were optimized as previously reported (Simirgiotis et al., 2016).

2.4 Fungal strains

In this study were included clinical isolated of yeasts (n=25) and filamentous fungi [non-dermatophytes (n=9) and dermatophytes (n=10)] and two standard strains from the American Type Culture Collection (ATCC), *Candida (C.) albicans* ATCC 64548 *and C. glabrata* ATCC 90030.

Yeasts strains: *C. albicans* (n=7), *C. tropicalis* (n=1), *C. guilliermondii* (n=3), *C. glabrata* (n=1), *C. dubliniensis* (n=1), *Candida* spp. (n=10), and *Saccharomyces (S.) cerevisiae* (n=2).

Non-dermatophyte filamentous fungi strains: *Aspergillus (A.) niger* (n=1), *A. clavatus* (n=1), *Penicillium (P.) islandicum* (n=1), *Penicillium* spp. (n=1), *Absidia (A.)* orchidis (n=1), *Mucor* spp. (n=1), *Rhizopus* spp. (n=1), *Alternaria* spp. (n=1), *Scopulariopsis (S.) brevicaulis* (n=1).

Dermatophyte filamentous fungi strains: *Trichophyton (T.) rubrum* (n=7); *T. mentagrophytes* (n=1), *Microsporum (M.) gypseum* (n=1) and *M. canis* (n=1).

Strains of yeasts and non-dermatophyte filamentous fungi, were obtained from the Servicio de Diagnóstico de la Cátedra de Micología, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Argentina; and the dermatophytes strains were obtained from the Servicio de Micología del Hospital Centro de Salud "Zenón Santillán" de San Miguel de Tucumán, Argentina.

The different clinical specimens were cultured on Sabouraud agar supplemented with chloramphenicol (0.05 g/L) (SA-Cl). All the strains were maintained in distilled water (Castellani, 1939; Castellani, 1963) and sub-cultured in SA-Cl every 15 days to prevent pleomorphic transformations.

2.5 Determination of Minimal Inhibitory Concentration (MIC)

MIC was defined as the lowest concentration of extracts capable to produce the total inhibition of the growth of fungi after incubation time. It was determined by two methods:

a) Macrodilution method in agar according to Derita et al., 2007 with some modifications. Briefly, stock solutions of extracts, diluted in ethanol 80% were added to the medium SA-Cl to give serial two-fold dilutions, resulting in concentrations ranging from 25 to 800 µg GAE/mL. The inoculum (2 µL) containing $5x10^4$ CFU/mL was spouted on top of the SA-Cl agar. Sterility control (no inoculum added), inoculum viability (no extract added), and solvent control (ethanol 80%), and positive control (Ketoconazole) were carried out.

Plates were incubated at 28 °C for 48 h for the case of yeasts, 72 h for non-dermatophyte filamentous fungi and 5 days for the dermatophytes strains. After incubation, fungal growth was evidenced by the presence of mycelium or visible colonies in the agar. For the resistent non-dermatophyte strains, the colony diameter (mm) was measured at 3, 7 and 10 days of development and the percentage inhibition of diameter growth (% IN) (Rosas-Burgos et al., 2009) was obtained according to the following formula:

$$\% IN = [(C - E)/ C] \times 100$$

where:

C = growth diameter in solvent control plate

E = growth diameter in a plate with extract

b) Microdilution method were based on the CLSI reference protocols M27-A3 (CLSI 2008a) and M38-A2 (CLSI 2008b), for yeasts and filamentous fungi, respectively. For the assay, stock solutions of extracts were two-fold diluted with medium SA-Cl (25 to 400 μg GAE/mL). A volume of 100 μL of inoculum suspension ($4x10^3$ CFU/mL) was added to each well.

Sterility, inoculum viability, solvent, and positive (Fluconazole) controls were included. Plates were incubated at 28 °C for 48 h for the case of yeasts, 72 h for non-dermatophyte filamentous fungi and 5 days for the dermatophytes strains. After incubation, fungal growth was evidenced by the presence of turbidity or a pellet on the well. The microdilution method was also used to determine MFC values.

2.6 Determination of minimal fungicidal concentrations (MFC)

MFC was defined as the lowest concentration of extracts capable to produce the irreversible inhibition of the viability of fungi after incubation time. To determine MFC values, after reading the corresponding MIC values, 10 μL of each well without visible growth were subcultured on SA-Cl plates. The plates were incubated at 28 °C for 48hs for the yeasts and 5 days for dermatophytes

fungi. The MFC values were determined as the lowest concentration of extracts where no visible growth was observed after incubation (Chellappandian et al., 2018).

All antifungal assays were tested in triplicate.

3. Results and discussion

3.1. Phytochemical analysis

In this work, a phytochemical study of *B. boliviensis* and *B. tola* hydroalcoholic extracts was conducted in order to obtain a standardized phytotherapeutic preparation of each plant species. *B. boliviensis* showed higher total phenolic and total flavonoids content than *B. tola* extract (Table 1). These results are in agreement with previous reports in these plant species collected in argentine Puna (Alberto et al., 2009; Sayago et al., 2012; Torres-Carro et al., 2015; Zampini et al., 2008). Calle et al., (2017) has reported similar content of total flavonoids in both plant species collected in Bolivia at 3600 masl.

The chromatographic patterns by UHPLC-MS of phytotherapeutic preparations of two *Baccharis* species were similar, both species present common chemical compounds (Figure 3, Table 2). Several compounds including terpenoids, flavonoids and phenolic acids have been identified in these two endemic South American *Baccharis* species for the first time (Table 2) using and orbitrap high resolution instrument. Some of the identified compounds have been previously identified and isolated. The detailed identification is explained above:

Phenolic and other organic acids

Peak 1 with a $[M-H]^-$ ion at m/z: 195.05072 was tentatively identified as gluconic acid ($C_6H_{12}O_7^-$), peak 2 with a $[M-H]^-$ ion at m/z: 191.01956 as quinic acid ($C_6H_7O_7^-$) and peaks 4 and 7 with a parent molecule at m/z: 367.10373 and 367.10376 producing fragments at m/z: 191.05573, and 134.03664 identified as feruloyl-quinic acids ($C_{17}H_{19}O_9^-$), the same case is peak 5 which was

appointed as chlorogenic acid ($C_{17}H_{17}O_9^-$), moreover, peaks 13-15 were identified as isomers of dicaffeoyl-quinic acid ($C_{25}H_{23}O_{12}^-$), (Simirgiotis et al., 2015), while peak 16 with a deprotonated molecule at m/z: 529.13519 was identified as 1-caffeoyl-5-feruloylquinic acid ($C_{26}H_{25}O_{12}^-$), finally, peak 27 was identified as syringaresinol ($C_{22}H_{25}O_8^-$) (Simirgiotis et al., 2016).

Flavonoids

Several compounds were identified as flavonols (UV max around 255-355 nm) others as flavanones (UV max at around 280 nm). Consequently, peak 6 with a $[M-H]^-$ ion at m/z: 593.15204 and producing a kaempferol ion at m/z: 285.05062 was identified as kaempferol-3-O-rutinoside ($C_{27}H_{29}O_{15}^-$). Peak 9 with a deprotonated molecule at m/z: 317.06656 was identified 3-hydroxy-hesperetin ($C_{16}H_{13}O_7^-$), peak 10-12 were identified as myricetin 7,3'-dimethyl ether ($C_{17}H_{13}O_8^-$), isorhamnetin ($C_{16}H_{11}O_7^-$) and palulitrin ($C_{22}H_{21}O_{13}^-$), while peaks 17, 18, 20, 21 and 23 were appointed as 8-prenyl-3-hydroxy-eriodictyol, 3-O-methyl-luteolin, eriodictyol, rhamnetin, and quercetin, respectively. Peaks 22, 26, 30, 32 and 36, with pseudomolecular ions at m/z: 389.08787, 373.09286, 375.07216, 359.07703 and 389.08832, and all producing myricetin fragment daughter ions at m/z: 317.03026 ($C_{15}H_9O_8^-$), were identified as myricetin 6-hydroxy-3, 7,3',5'-tetramethyl ether, myricetin 8-methyl-7,3',5'-trimethyl ether, myricetin 6-hydroxy-7,3',5'-trimethyl ether, myricetin 7,3',5'-trimethyl ether and myricetin-6,7,3',5'-tetramethyl ether, respectively; some of those compounds have been previously isolated (Zampini et al., 2009a). Peak 28 was identified as the acetyl derivative of myricetin. In the same manner, peak 19, 24 and 31, producing isorhamnetin ions at m/z: 315.05102 ($C_{16}H_{11}O_7^-$), were identified as the 3-O-methyl and 7-O-methyl ether of isorhamnetin, and the 3,7-di-O-methyl ether derivative, respectively, methoxylated compounds coincident with those isolated previously (Zampini et al., 2009a). Peak 25 with at m/z: 345.06186 was identified as syringetin ($C_{17}H_{13}O_8^-$), peak 44 was identified as 5,7,3',4'-tetrahydroxy-6-methoxyflavanone; filifolin, and peak 43 at m/z: 375.07670 was identified

as limocitrol ($C_{18}H_{15}O_9^-$). MS spectra and structures of some methoxylated flavones detected in *Baccharis* species are shown in Figure 4 and Figure S1.

Diterpenoids

Peak 34 with a pseudomolecular ion at m/z: 301.1807 was identified as the clerodane diterpenoid solidagoiol A ($C_{20}H_{29}O_2^-$), (Simirgiotis et al., 2016) a reduction derivative of solidagoic acid A (MW: 315.1966, $C_{20}H_{27}O_3^-$, not detected) while peak 29 with a pseudomolecular ion at m/z: 373.20205 as 19-acetoxy-solidagoic acid A ($C_{22}H_{29}O_5^-$), peak 33 as 19-hydroxy-solidagoiol A, and peak 35 with a $[M-H]^-$ ion at m/z: 359.22276 as 19-O-acetyl-soligagoiol A ($C_{22}H_{31}O_4^-$), and peak 45 as 3,4 dihydro-19-acetoxy-solidagoic acid A ($C_{22}H_{31}O_5^-$), (Simirgiotis et al., 2016) while peak 37 was identified as hawtriwaic acid ($C_{20}H_{27}O_4^-$) (Simirgiotis et al., 2000) and peaks 47 and 48 with pseudomolecular ions at m/z: 287.20142 ($C_{20}H_{31}O^-$) were identified as ent-Beyer-15-en-18-ol compounds previously reported in *B. tola* (Parra et al., 2015; San Martín et al., 1980;Simirgiotis et al., 2016). Peaks 38 and 39, with pseudomolecular ions at m/z: 433.23345 and 433.22263 Daltons were identified as the isomers: 17-O-succynyl-3,7-dihydroxy-ent-cleroda-1,13(16),14-triene-15,16-oxide, and 17-O-succynyl-3,4-dihydroxy-ent-cleroda-1,13(16),14-triene-15,16-oxide ($C_{24}H_{33}O_7^-$) (Zdero et al., 1989), in the same manner peaks 40 and 41 were identified as 17-O-succynyl-3-hydroxy-ent-cleroda-1,13(16),14-triene-15,16-oxide and 17-O-succynyl-4-hydroxy-ent-cleroda-1,13(16),14-triene-15,16-oxide, and peak 42 as 17-O-succynyl-3,4,7-trihydroxy-ent-cleroda-1,13(16),14-triene-15,16-oxide ($C_{24}H_{33}O_8$) while peak 46 was identified as 17-O-succynyl-ent-cleroda-1,13(16),14-triene-15,16-oxide and finally peak 49 as 17-O-succynyl-ent-cleroda-1,13(16),14-triene-15,16-oxide ($C_{26}H_{37}O_7^-$). Some of those compounds were previously isolated from *B. boliviensis* (Zdero et al., 1989). MS spectra and structures of some clerodanes detected in *Baccharis* species are shown in Figure S1

Other compounds

Peak 3 with a $[M-H]^-$ ion at m/z: 205.07159 as diethyl tartrate ($C_8H_{14}O_6^-$), Peak 8 was identified as barlerin ($C_{19}H_{27}O_{12}^-$), Peak 52 at m/z: 289.11185 was identified as the coumarin sibiricin ($C_{16}H_{17}O_5^-$), peaks 50 and 51 with ions at m/z: 325.18453 and 339.20023 were tentatively identified as heptaethylene glycol and heptaethylene glycol methyl ether, respectively.

3.2. Antifungal activity of Baccharis extracts

Phytotherapeutic preparations were tested against a panel of fungal strains which included yeasts (*Candida* spp. and *Saccharomyces cerevisiae*) and filamentous fungi (non- dermatophytes and dermatophytes) which were selected for their clinical relevance. *C. albicans* and *C. tropicalis* were selected due to their high incidence of candidemia (more than 80%) in Latin America (Nucci et al., 2013). *C. glabrata* has been identified as the second leading cause of adult candidemia particularly in patients with hematologic malignancies (Pfaller and Diekema, 2007). Among filamentous fungi, *Fusarium* spp., *Aspergillus* spp., and *S. brevicaulis* are the most commonly recovered in opportunistic mycoses following *Candida* spp. (Babayani et al., 2018). Cutaneous fungal infections are among the most common fungal infections and are mainly caused by keratinophilic filamentous fungi (dermatophytes), that use keratin as a nutrient in skin, hair and nail infections (Massiha and Muradoy, 2015). Dermatophyte fungi included *T. rubrum*, *T. mentagrophytes*, *M. gypseum* and *M. canis* due to their high incidence in superficial fungal infections (Ayatollahi Mousavi and Kazemi, 2015). Species of *Trichophyton* account for as many as 70% of all dermatophyte infections (Jarabrán et al., 2015; Mukherjee et al., 2003); *M. gypseum*, although saprophytic in immunocompetent individuals, produces important infections in immunocompromised patients due to its keratinolytic potential (Tambekar et al., 2007).

The *in vitro* antifungal assay (MIC and MFC values) of ethanolic extracts of *B. boliviensis* and *B. tola* was performed using macrodilution and microdilution methods. The results revealed that in general, the assayed dermatophyte isolates, were more sensitive to the preparation of *B. boliviensis*

than to that of *B. tola*. Moreover, *C. guilliermondii, C. dubliniensis,* and *S. cerevisiae* were the species most sensitive to both phytotherapeutic preparations, with MIC values between 50 and 200 µg GAE/mL for *B. boliviensis*, and between 200 and 400 µg GAE/mL for *B. tola* (Table 3). Within the filamentous fungi, the viability of non-dermatophyte fungi were not affected by the preparations while the dermatophyte fungi strains were the most sensitive isolates to both extracts; thereby 100% of the strains were inhibited by *B. boliviensis* preparation with MIC values between 25 and 200 µg GAE/mL and MFC values between 50 and 400 µg GAE/mL whereas that of *B. tola* inhibited the 80% of the strains with MIC values between 100 and 400 µg GAE/mL and MFC values between 200 and 400 µg GAE/mL. *T. rubrum* isolates were the most sensitive strains (Table 3). These results are interesting, considering that *T. rubrum* is the etiological agent of 80–93% of all clinical infections produced by dermatophytes. Moreover, all dermatophytes strains were resistant to fluconazole (positive control), with MIC values > 128 µg/mL; it has been reported that certain strains of dermatophytes are resistant to fluconazole (Méndez-Tovar, et al., 2007; Santos and Hamdan, 2006). In general, the most frequently systemic antifungal agents used for dermatophytosis treatment are fluconazole, terbinafine, and itraconazole, with prolonged schedules, risk of drug interactions and high associated costs, which hinder adherence to treatment; in most cases the success obtained with the treatment used is low and it may be due to multiple factors, including the resistance of isolated fungi to the medications used (Gupta et al., 2001).

Furthermore, the percentage inhibition of diameter growth (% IN) produced by each extract was determined against the non-dermatophyte strains that were resistant to the extracts in the highest tested concentration (800 µg GAE/mL) in order to find out the effect of each extract on the mycelium growth. Table 4 shows the results obtained with 3200 µg GAE/mL. *S. brevicaulis* was the strain more inhibited by the hydroalcoholic extracts, showing 100% inhibition with *B. boliviensis* after ten days of incubation while *B. tola* inhibit the growth up to seven days, and after

ten days of incubation just 10% of growth was observed. *A. orchidis*, *Alternaria* spp. and *Mucor* spp. showed also a strong inhibition by both extracts. *A. niger*, *A. clavatus* and *P. islandicum* were the more resistant strains (Table 4). In Figure 5 a representative photo of the filamentous fungi growth inhibition produced by the hydroalcoholic extracts of *B. boliviensis* and *B. tola* is shown.

The presence of several identified compounds in this work would support the *Baccharis* extracts antifungal activity. Several flavonoids identified have been regarded as antifungal agents (Afifi et al., 1991; Candiracci et al., 2011; Santos and Pereira, 2018; Weidenbörner and Jha, 1997) in particular, some methylated flavones (Figure 4 and Figure S1) could be of particular attention due to more liposolubility (Meragelman et al., 2005). On the other hand, clerodanes diterpenes are bioactive compounds isolated from several plant species with several biological properties reported, including antifungal activity (Li et al., 2016). For instance, three neo-clerodane diterpenoids, jodrellin A, jodrellin B and clerodin isolated from *Scutellaria* showed antifungal activity against the pathogenic fungi *Fusarium oxysporum* f. sp. lycopersici and *Verticillium tricorpus* (Cole et al., 1991). According the analysis of UHPLC-OT-MS metabolome fingerprinting, some ent-clerodanes are present in *B. boliviensis* extract (peak 40, 41 and 42) but not in *B. tola* which could be related to the greater antifungal activity of *B. boliviensis*. Regarding ent-clerodane terpenoids from *Baccharis* and antifungal activity, also some ent-clerodanes demonstrated antifungal activity in a synergistic manner combined with antifungal drugs such as terbinafine (Rodriguez et al., 2013, Rodriguez et al., 2019).

Previous studies performed with ethanolic extract of *B. boliviensis* from Monte region of Argentina (Mendoza province, at 1890 masl), did not demonstrated antifungal activity against yeast (*C. albicans*, *C. tropicalis*, *Cryptococcus neoformans*, *S. cerevisiae*), filamentous fungi *(A. fumigatus*, *A. flavus*, *A. niger*) nor dermatophytes (*M. gypseum*, *T. rubrum*, *T. mentagrophytes* and *Epidermophyton floccosum*) at a concentration of 1000 μg/mL (Svetaz et al., 2010). Probably the

chemical composition of plants collected in different eco-regions from Argentina (Monte region vs Puna region) presents some differences. However, the chemical composition of *B. boliviensis* extract from Mendoza province was not reported until now. Ethanolic extracts of other plant species from the extremophile region of the Puna (*Chuquiraga atacamensis*, *Parastrephia phyliciformis*, *Parastrephia lepidophylla* and *Tetraglochin andina*) presented antifungal activity on mycelial phytopathogenic fungi and vaginal yeast species (with MIC values of 250-500 µg GAE/mL) (Moreno et al., 2018a; Palavecino Ruiz et al., 2016; Sayago et al., 2012).

Other *Baccharis* species that growth in Argentina such as *B. darwinii* and *B. articulate* showed activity against dermatophytes fungi (*T. rubrum, T. mentagrophytes* and *M. gypseum*) with MIC values of 250-500 µg/mL and MFC values of 100-500 µg/mL, but did not present activity (MIC > 1000 µg/mL) against yeasts and/or non-dermatophyte fungi (*C. albicans*, *C. tropicalis, S. cerevisiae*, *Cryptococcus neoformans, A. flavus, A. fumigatus, A. niger*) (Kurdelas et al., 2010; Vivot et al., 2009). On the other hand, other authors reported that *B. pingraea, B. medullosa, B. notosergila, B. polifolia, B. stenophylla, B. articulate, B. crispa, B. gaudichaudiana, B. microcephala, B. penningtonii, B. phyteumoides, B. sagittalis, B. triangularis, B. trimera* and *B. grisebachii*, did not present activity against dermatophytes, yeasts and/or filamentous fungi (*C. albicans, C. tropicalis, C. neoformans*, S. cerevisiae, *A. fumigatus, A. flavus, A. niger, M. gypseum, T. rubrum, T. mentagrophytes, E. floccosum*), up to 1000 µg/mL (Feresin et al., 2001; Rodriguez et al., 2013; Svetaz et al., 2010).

Rosas-Burgos et al. (2009) reported the effect of methanolic extract of *Baccharis glutinosa* (8.4 mg/mL) against filamentous fungi (*A. flavus, A. parasiticus*, and *F. verticillioides*) with an inhibition of no more than 60% after 14 days of incubation.

4. Conclusion

The phytotherapeutic preparations obtained with aerial parts of *B. tola* and *B. boliviensis*, two shrubs growing in the Puna, are used popularly to treat skin and soft tissue infections by topical application. The results indicate that the hydroalcoholic preparations obtained from aerial parts of *B. boliviensis* and *B. tola* could be used for the treatment of infectious skin disease produced by fungi, principally those caused by dermatophytes, the most common responsible of infectious skin disease that requires a long time treatment. Fifty-two compounds including several terpenoids and poly-hydroxilated and methylated flavonoids were accurately identified from *B. boliviensis* and *B. tola* for the first time, and several of identified compounds are regarded as antifungal agents. Further investigations are being carried out in our laboratory to isolate and characterize the active components of the plant extracts.

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Figure legends

Figure 1. Map of the Argentine Puna, the collection site of plants (Antofagasta de la Sierra, Catamarca, Argentina).

Figure 2. Photography of plants species: **A)** and **B)** *B. boliviensis;* **C)** and **D)** *B. tola*..

Figure 3. UHPLC-MS Chromatograms of a) *B. boliviensis* extract (Base peak, negative mode) and b) *B. tola* extract (Base peak, negative mode).

Figure 4. Examples of methoxylated flavones detected in *Baccharis* species

Figure 5. Representative photo of radial growth inhibition of filamentous fungi at 7 days of development. **A)** *Mucor* spp. **B)** *Absidia orchidis*. **C)** *Alternaria* spp. **D)** *Penicillium* spp. **1)** Solvent control (80% ethanol), **2)** *B. boliviensis* hydroalcoholic extract (3200 μg GAE/mL). **3)** *B. tola* hydroalcoholic extract (3200 μg GAE/mL).

Fig. S1. Examples of methoxylated flavones and clerodanes detected in *Baccharis* species. Full MS spectra and structures of compounds 10, 30, 38, 42, 44 and 46.

Table 1: Total phenolic compounds and total flavonoids content of *B. boliviensis* and *B. tola* hydroalcoholic extracts

Sample	Total phenolic (µg GAE/mL)	Total flavonoids (µg QE/mL)
B. boliviensis	3780.6 ± 19.6	1354.5 ± 131.9
B. tola	2441.7 ± 286.8	402.9 ± 66.9

Table. 2. High resolution UHPLC-PDA-MS metabolite profiling data of the phytotherapeutic preparation of *Baccharis boliviensis* and *B. tola* from Puna.

Peak #	Retention time (min.)	UV max (nm)	Tentative identification	Elemental composition $[M-H]^-$	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Accuracy (δppm)	MS^n ions (*m/z*)	Species€
1	2.84	-	Gluconic acid	$C_6H_{12}O_7^-$	195.04993	195.05072	0.22		a,b
2	2.96	-	Quinic acid	$C_6H_7O_7^-$	191.01863	191.01956	4.88		b
3	10.98	-	Diethyl tartrate	$C_8H_{14}O_6^-$	205.07066	205.07159	4.53		b
4	11.32	210	Feruloyl-quinic acid	$C_{17}H_{19}O_9^-$	367.10236	367.10373	3.73	191.05573, 134.03664	a
5	11.96		Chlorogenic acid*	$C_{17}H_{17}O_9^-$	356.08671	353.08826	4.38	707.18463 ($2M-H^-$), 191.05603 (quinic moiety)	b
6	12.50	265-365	Kaempferol-3-O- rutinoside	$C_{27}H_{29}O_{15}^-$	593.15010	593.15204	4.38	285.05062 ($C_{15}H_9O_6^-$)	a,b
7	12.63	210	Feruloylquinic acid	$C_{17}H_{19}O_9^-$	367.10236	367.10376	3.81	191.05577, 134.03664	a,b

8	12.95	210	Barlerin	$C_{19}H_{27}O_{12}^-$	447.14970	447.15118	3.31	
9	13.25	285	3-Hydroxy-hesperetin	$C_{16}H_{13}O_7^-$	317.06668	317.06656	0.31	302.04291 ($C_{15}H_{10}O_7^-$)	a,b
10	13.50	277-353	Myricetin 7,3'-dimethyl ether	$C_{17}H_{13}O_8^-$	345.0616	345.06152	0.28	330.0377, 315.01445	b
11	13.75	252-352	Isorhamnetin*	$C_{16}H_{11}O_7^-$	315.05090	315.05103	-0.3	301.03535, 179.03342, 151.05463	a,b
12	13.96	252-352	Palulitrin	$C_{22}H_{21}O_{13}^-$	493.09767	493.09909	2.88		a,b
13	14.27	213, 287, 326	1,3- Di-O-caffeoyl quinic acid	$C_{25}H_{23}O_{12}^-$	515.11840	515.11975	2.61	191.05585	a
14	14.47	213, 287, 326	1,4- Di-O-caffeoyl quinic acid	$C_{25}H_{23}O_{12}^-$	515.11840	515.11969	2.49	191.055853	a,b
15	14.88	213, 287, 326	1,5- Di-O-caffeoyl quinic acid	$C_{25}H_{23}O_{12}^-$	515.11840	515.11987	2.85	191.055852	a,b
16	15.32	252-352	1-O-Caffeoyl-5-O-feruloylquinic acid	$C_{26}H_{25}O_{12}^-$	529.13405	529.13519	2.15	191.05577 (quinic acid), 134.03664	a,b
17	16.05	286-325	8-Prenyl-3-hydroxy-eriodictyol	$C_{20}H_{19}O_7^-$	371.11363	371.11331	0.80	239.10718 ($C_{16}H_{15}O_2$-)	a,b
18	17.04	254-354	3-*O*-Methyl-luteolin	$C_{16}H_{11}O_6^-$	299.05611	299.05591	0.66	284.03220 ($C_{15}H_8O_6^-$)	a
19	17.27	252-352	Isorhamnetin 3-methyl ether	$C_{17}H_{13}O_7^-$	329.06668	329.02998	11.17	315.05102 ($C_{16}H_{11}O_7^-$) ()314.0065, 286.0115 ($C_{14}H_6O_7^-$)	b
20	17.95	280	Eriodictyol*	$C_{15}H_{12}O_6^-$	287.05501	287.05643	4.94	179.05342	a,b
21	18.02	254-355	Rhamnetin	$C_{16}H_{11}O_7^-$	315.04993	315.05136	4.54	301.03548, 179.03832, 151.05478	a,b
22	18.32	277-353	Myricetin 6-hydroxy-3, 7,3',5'-tetramethyl ether	$C_{19}H_{17}O_9^-$	389.08781	389.08787	0.02	359.04041 ($C_{17}H_{11}O_9^-$), 317.03026 ($C_{15}H_9O_8^-$)	a,b
23	19.51	277-353	Quercetin*	$C_{15}H_9O_7^-$	301.03428	301.03574	4.84	271.83310, 177.09688	a,b
24	19.72	252-352	Isorhamnetin 7-methyl ether	$C_{17}H_{13}O_7^-$	329.06668	329.06644	0.37	315.05102 ($C_{16}H_{11}O_7^-$), 299.01947 ($C_{15}H_7O_7^-$)	a,b

25	21.37	252-352	Syringetin	$C_{17}H_{13}O_8^-$	345.06234	345.06186	3.51	301.03528, 179.03342, 151.05476	a,b
26	21.52	277-353	Myricetin 8-methyl-7,3',5'-trimethyl eter	$C_{19}H_{17}O_8^-$	373.09277	373.09286	-0.08	343.04562 ($C_{17}H_{11}O_8^-$), 317.03026 ($C_{15}H_9O_8^-$)	a,b
27	21.63	248-272sh	Syringaresinol	$C_{22}H_{25}O_8^-$	417.15553	417.11895	8.77	407.0903, 371.1135 [M-H-HCOOH]-	a,b
28	21.75	277-353	3-Acetyl-myricetin	$C_{17}H_{11}O_9^-$	359.04086	359.04047	1.11	317.03026 ($C_{15}H_9O_8^-$)	b
29	21.86	205	19-Acetoxy-solidagoic acid A	$C_{22}H_{29}O_5^-$	373.20192	373.20205	1.21		
30	21.97	277-353	Myricetin 6-hydroxy-7,3',5'-trimethyl ether	$C_{18}H_{15}O_9^-$	375.07211	375.07216	0.03	345.02481 ($C_{16}H_9O_9^-$), 317.03026 ($C_{15}H_9O_8^-$)	a,b
31	22.12	252-352	Isorhamnetin 3,7-di-methyl ether	$C_{18}H_{15}O_7^-$	343.08233	343.08221	-0.26	315.05102 ($C_{16}H_{11}O_7^-$) 313.03506 ($C_{16}H_9O_7^-$)	a,b
32	22.56	277-353	Myricetin 7,3',5'-trimethyl ether	$C_{18}H_{15}O_8^-$	359.07724	359.07703	0.27	313.03506 ($C_{16}H_9O_7^-$), 317.03026 ($C_{15}H_9O_8^-$)	a,b
33	22.67	205	19-Hydroxy-Solidagoiol A	$C_{20}H_{29}O_3^-$	317.21224	317.21204	0.63		a,b
34	23.12	203	Solidagoiol A	$C_{20}H_{29}O_2^-$	301.21738	301.18073	10.52		b
35	23.43	203	19-*O*-acetyl-soligagoiol A	$C_{22}H_{31}O_4^-$	359.22287	359.22276	0.27		b
36	23.87	277-353	Myricetin -6, 7,3',5'-tetramethyl ether	$C_{19}H_{17}O_9^-$	389.087815	389.08832	0.77	359.04041 ($C_{17}H_{11}O_9^-$), 317.03026 ($C_{15}H_9O_8^-$)	a
37	23.62	204	Hawtriwaic acid	$C_{20}H_{27}O_4^-$	331.19146	331.19157	-0.31	301.18060 ($C_{16}H_{15}O_2^-$, -CH_2OH)	a,b
38	24.10	220	17-O-succynyl-3,7-dihydroxy-ent-cleroda-1,13(16),14-triene-15,16-oxide	$C_{24}H_{33}O_7^-$	433.22208	433.23345	1.26		a,b
39	24.63	220	17-O-succynyl-3,4-dihydroxy-ent-cleroda-1,13(16),14-triene-15,16-oxide	$C_{24}H_{33}O_7^-$	433.22208	433.22263	3.16		a,b
40	24.67	220	17-O-succynyl-3-hydroxy-ent-cleroda-1,13(16),14-triene-15,16-oxide	$C_{24}H_{33}O_6^-$	417.22717	417.22864	3.52		a

41	24.73	220	17-O-succynyl-4-hydroxy-ent-cleroda-1,13(16),14-triene-15,16-oxide	$C_{24}H_{33}O_6^-$	417.22717	417.22867	3.60		a
42	24.85	220	17-O-succynyl-3,4,7-trihydroxy-ent-cleroda-1,13(16),14-triene-15,16-oxide	$C_{24}H_{33}O_8^-$	449.21699	449.21857	3.52		a
43	24.92	254 356	Limocitrol	$C_{18}H_{15}O_9^-$	375.07673	375.07670	2.12	258.17055, 179.05436	b
44	25.40	254-355	5,7,3',4'-tetrahydroxy-6-methoxyflavanone; Filifolin	$C_{16}H_{13}O_7^-$	317.06558	317.06689	4.14		a
45	25.93	205	3,4 Dihydro-19-acetoxy-solidagoic acid A	$C_{22}H_{31}O_5^-$	375.21660	375.21741	2.14		a
46	26.25	220	17-O-succynyl-ent-cleroda-1,13(16),14-triene-15,16-oxide	$C_{24}H_{33}O_5^-$	401.23225	401.23380	3.84		a,b
47	26.42	-	*ent*-Beyer-15-en-18-ol	$C_{20}H_{31}O^-$	287.23805	287.20142	12.7		a,b
48	26.76	-	*ent*-Beyer-15-en-19-ol	$C_{20}H_{31}O^-$	287.23806	287.20153	12.8		a
49	27.86	220	17-O-succynyl-3,7-dimethoxy-ent-cleroda-1,13(16),14-triene-15,16-oxide	$C_{26}H_{37}O_7^-$	461.25338	461.25497	3.45		a
50	28.23	-	Heptaethylene glycol	$C_{14}H_{3O}O_8^-$	325.18569	325.18453	-3.52		a
51	28.45	-	Heptaethylene glycol methyl ether	$C_{15}H_{31}O_8^-$	339.20134	339.20023	-3.22		a,b
52	29.10	270-310	Sibiricin	$C_{16}H_{17}O_5^-$	289.11292	289.11185	-3.7		a

*Compounds identified with spiking experiments with authentic standards. [€]*a: Baccharis boliviensis and b: B. tola*

Table 3: MIC, MFC values of the hydroalcoholic extracts and MIC values of ketoconazole or fluconazole.

Strains	Macrodilution (MIC) (µg GAE/mL)		Microdilution (MIC/MFC) (µg GAE/mL)		Ketoconazole (µg/mL)
	B. boliviensis	*B. tola*	*B. boliviensis*	*B. tola*	
Yeasts					
C. albicans A1	R	R	R	R	2
C. albicans A2	R	R	R	R	8
C. albicans A3	800	R	R	R	0.12
C. albicans A4	R	R	R	R	2
C. albicans A5	R	R	R	R	8
C. albicans A6	R	R	R	R	0.12
C. albicans A7	R	R	R	R	0.5
C. glabrata B1	R	R	R	R	16
C. guilliermondii C1	100	400	100/R	400/R	0.03
C. guilliermondii C2	50	400	50/R	400/R	0.03
C. guilliermondii C3	800	R	R	R	0.12
C. tropicalis D1	R	R	R	R	4
C. dubliniensis E1	100	800	100/R	R	-
Candida spp. F1	R	R	R	R	0.12
Candida spp. F2	R	R	R	R	0.06
Candida spp. F3	R	R	R	R	0.06
Candida spp. F4	R	R	R	R	2
Candida spp. F5	R	R	R	R	16
Candida spp. F6	R	R	R	R	0.5
Candida spp. F7	R	R	R	R	0.5
Candida spp. F8	R	R	R	R	0.5
Candida spp. F9	R	R	R	R	0.5
Candida spp. F10	R	R	R	R	0.5
S. cerevisiae G1	200	200	200/R	200/R	16
S. cerevisiae G2	800	800	R	R	1
C. albicans ATCC 64548	R	R	R	R	8
C. glabrata ATCC 90030	R	R	R	R	4
Dermatophytes					Fluconazole
T. rubrum 014	100	400	100/100	400/400	> 128
T. rubrum 006	100	400	100/100	400/400	> 128
T. rubrum 067	100	200	100/100	200/200	> 128
T. rubrum 020	25	100	25/50	100/200	> 128
T. rubrum 105	200	200	200/200	200/R	> 128
T. rubrum 102	50	200	50/200	200/400	> 128
T. rubrum 099	100	200	100/200	200/R	> 128

T. mentagrophytes	200	800	200/400	R	> 128
M. gypseum	200	800	200/200	R	> 128
M. canis 108	100	200	100/100	200/R	> 128
Non-dermatophytes					Ketoconazole
A. niger	R	R	R	R	-
A. clavatus	R	R	R	R	-
Penicillium spp.	R	R	R	R	-
P. islandicum	R	R	R	R	-
A. orchidis	R	R	R	R	4
Mucor spp.	R	R	R	R	4
Rhizopus spp.	R	R	R	R	-
Alternaria spp.	R	R	R	R	> 8
S. brevicaulis	R	R	R	R	> 8

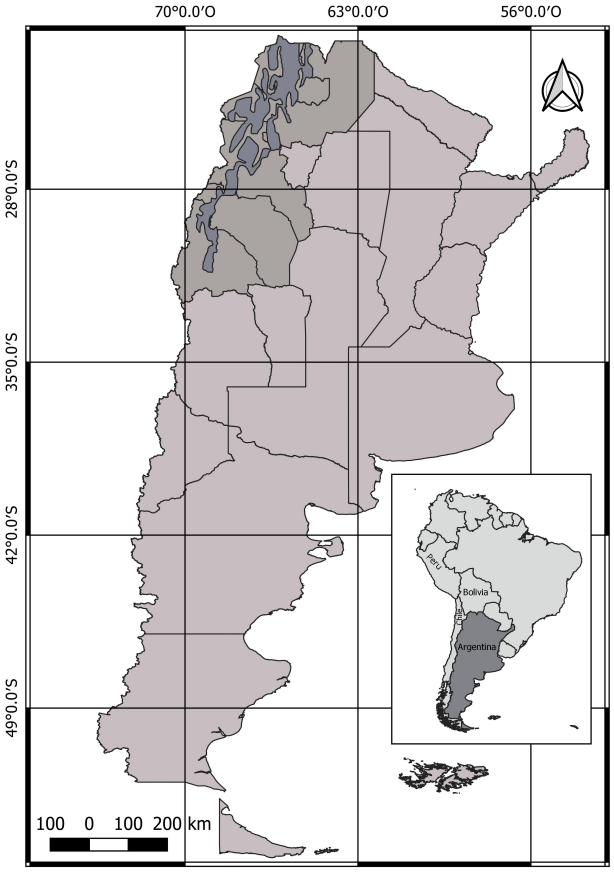
-: not tested

R= resistant until the highest concentration tested (macrodilution: 800 µg GAE/mL; microdilution: 400 µg GAE/mL).

Table 4: Radial growth inhibition of hydroalcoholic extracts (3200 µg GAE/mL) against Non-dermatophytes strains.

Strains	Growth inhibition (%)*					
	B. boliviensis			*B. tola*		
	3 days	7 days	10 days	3 days	7 days	10 days
A. niger	26	0	0	7.3	0	0
A. clavatus	33.3	10.6	9	24.9	10.6	9
Penicillium spp.	28.8	33.5	39	28.8	33.5	39.1
P. islandicum	0	23.3	33.3	0	23.3	16.7
A. orchidis	100	87.4	58.4	75	67.4	51.6
Mucor spp.	84.4	77.8	20	80	75	0
Rhizopus spp.	0	66.7	30	0	66.7	30
Alternaria spp.	100	100	100	90	78	60
S. brevicaulis	100	100	100	100	100	90

* The percentage of radial growth inhibition was calculated as % IN= [(C – E)/ C] X100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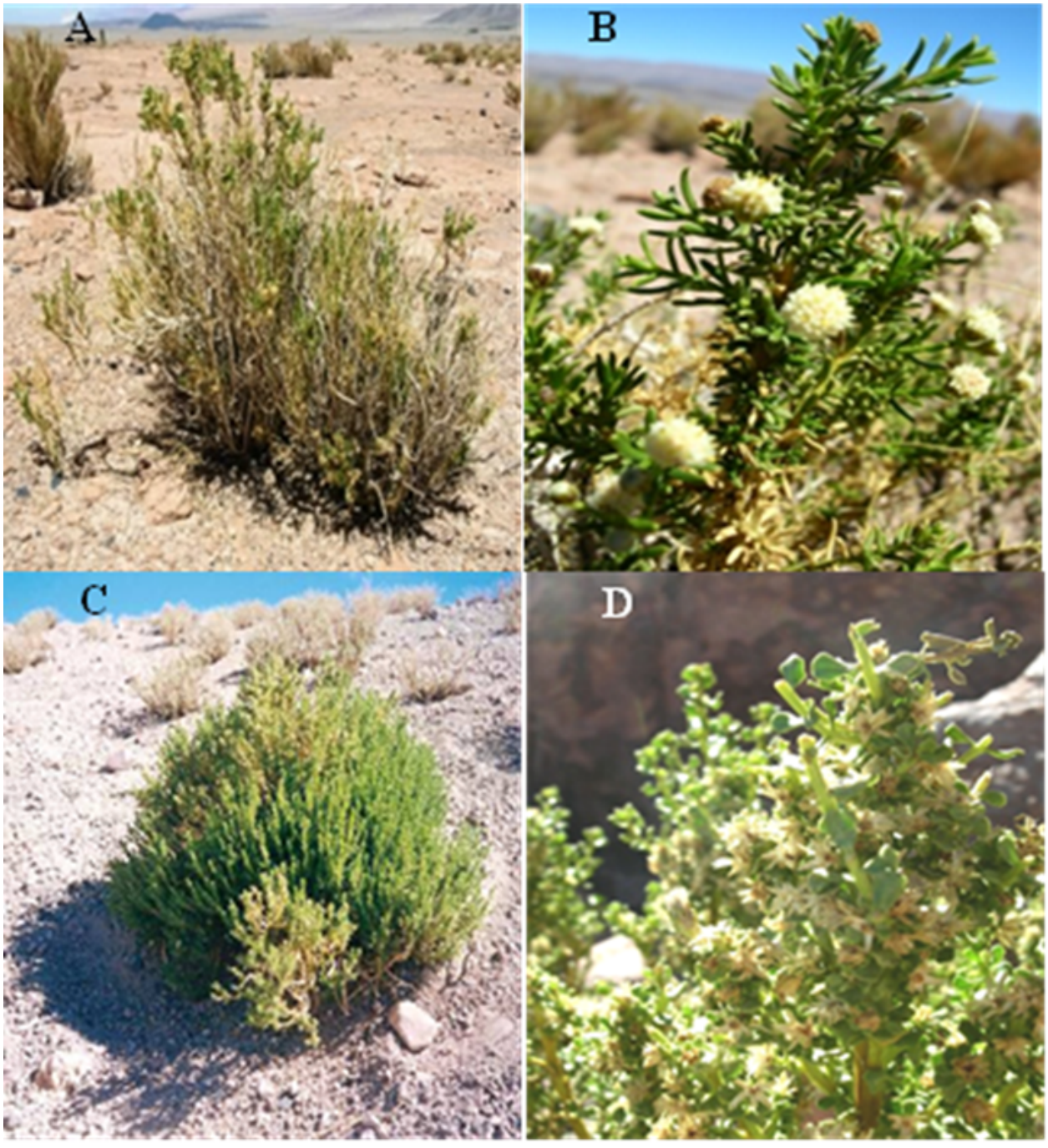
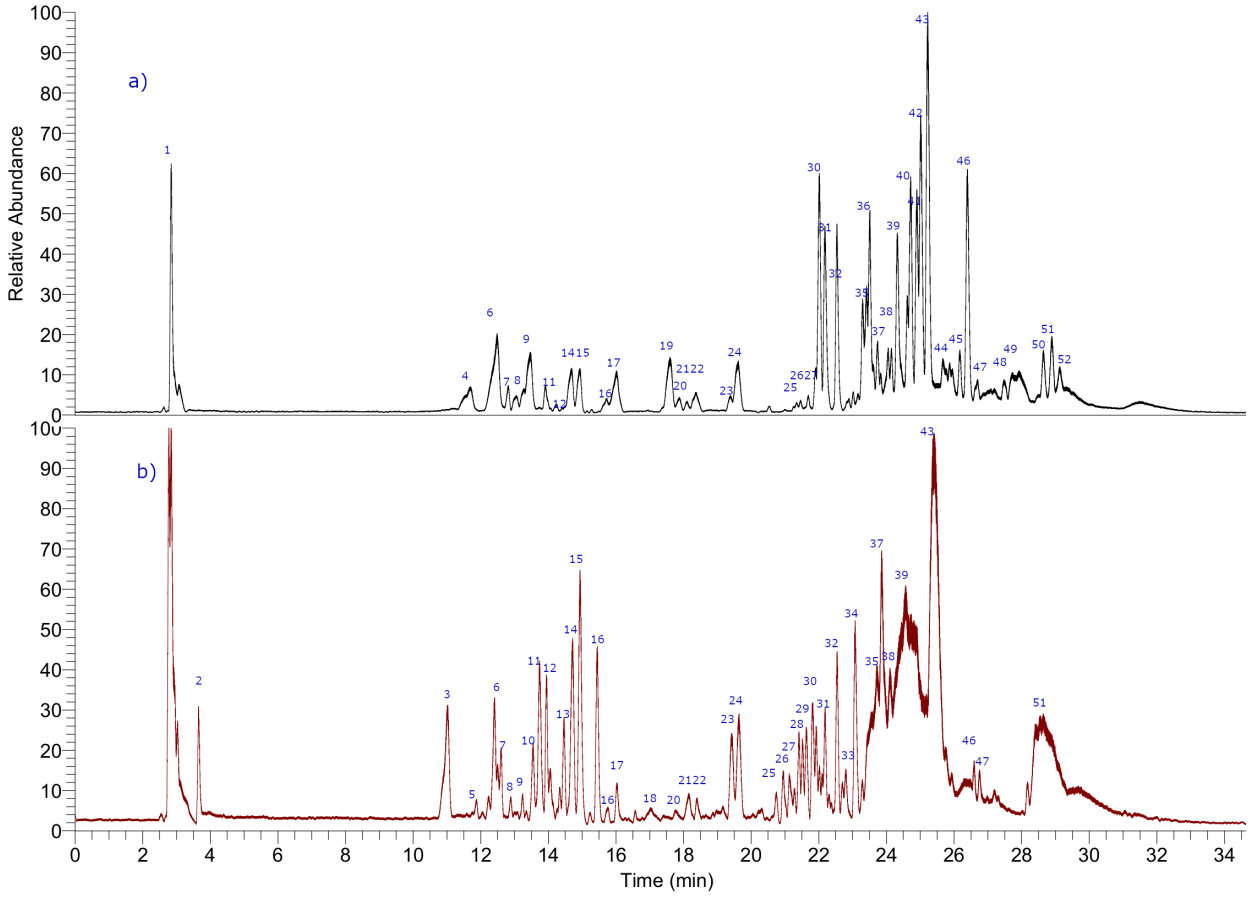
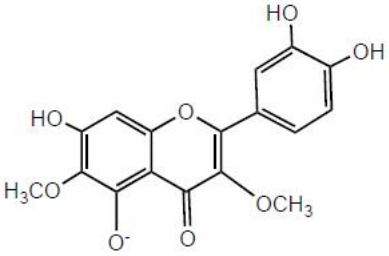
28°0.0'S
35°0.0'S
42°0.0'S
49°0.0'S
Peru
Bolivia
Chile
Argentina
100 0 100 200 km

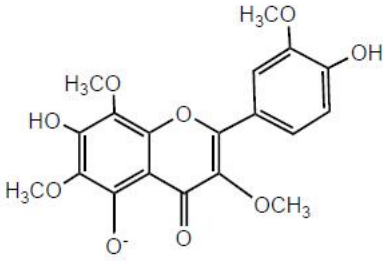

Jujuy
Salta
Antofagasta
de la Sierra
Catamarca
La Rioja
San Juan


A
B
C
D

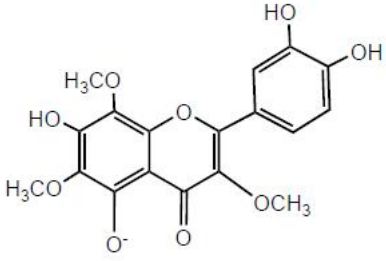




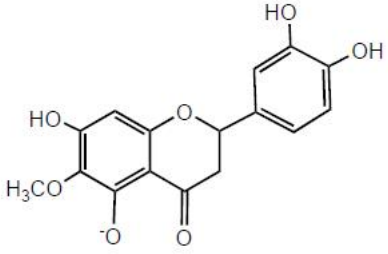
Peak 10: $C_{17}H_{13}O_8^-$
Exact Mass: 345,06159



Peak 22: $C_{19}H_{17}O_9^-$
Exact Mass: 389,08781



Peak 30: $C_{18}H_{15}O_9^-$
Exact Mass: 375,07216



Peak 44: $C_{16}H_{13}O_7^-$
Exact Mass: 317,06668