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Research Paper

Phenolic profile, chemical relationship and antifungal activity of Andean *Hypericum* species

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ABSTRACT

Fungal infections represent a major and increasing threat to public health resulting in a growing demand for new drugs. The genus *Hypericum* is a rich source of bioactive secondary metabolite, including antimicrobials and the exploration of uninvestigated species holds the potential for the discovery of novel molecules for the pharmaceutical market. Here the chemical composition, phytochemical relationships and the antifungal potential of seven of neglected Andean *Hypericum* species has been investigated. Through liquid chromatography and mass spectrometry analysis, 34 phenolic compounds have been identified and quantified. Quercetin derivatives, catechin and epicatechin, procyanidin B2 and chlorogenic acid derivatives represented the most abundant compounds in all extracts. The antifungal activity was evaluated against a panel of clinical isolates of pathogenic fungi. Four of the tested extracts were active against *C. albicans* (MIC_{50} < 353.25 µg/ml) and five against *C. parapsilosis* (MIC_{50} < 1000 µg/ml). Noteworthy was the activity of *H. garciae,* that was subjected to a comprehensive investigation against a broad panel of *Candida* spp. clinical strains. *H. garciae* extracts showed higher activity than fluconazole against *C. intermedia* and *C. parapsilosis*. The methanolic extract was active against all of the tested species with MIC_{50} values as low as 4 µl/ml and 5 µl/ml against *C. lusitaniae* and *C. albicans* respectively and 64 µl/ml against *C. glabrata.* The chloroformic extract displayed a higher activity than fluconazole against *C. tropicalis*. The presented data represent an advancement in the knowledge of the chemistry and antifungal potential of *Hypericum* species and offer interesting suggestions for deeper studies in the field of chemotaxonomy and drug discovery.

1. Introduction

Fungal infections represent a major and increasing threat to public health. The risk of invasive fungal disease is especially high in immunocompromised individuals, causing a higher rate of mortality than tuberculosis or malaria (Lass-Floerl, 2009). *Candida albicans* is the most commonly encountered pathogen of invasive candidiasis, a serious disease that can affect various parts of the body (e.g. blood, heart, brain) (Rizzetto et al., 2015), yet over the past 25 years also non-*albicans* species, like *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. lusitaniae*, have been frequently detected (Pfaller et al., 2014). Despite the continuous search for new antifungals and the introduction of novel antimycotic drugs, invasive candidiasis still represents a major challenge for medicine. This situation is worsened by the alarming fact that the emergence of drug resistance is more common in *non-albicans Candida* as compared with *C. albicans* (Cornely et al., 2014). Consequently, the search for new antifungal therapies and lead molecules is of prime importance for the pharmaceutical industry.

During the past two decades, an interest in the isolation of antimicrobial compounds from plants has been reawakened (Abreu et al., 2012). Plants have traditionally provided remedies for human health, and it is estimated that approximately 25% of drugs used in clinical

Abbreviations: MIC_{50}, minimal inhibitory concentration required to inhibit the growth of 50% organisms; GM, geometric mean

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settings are based on plant-derived molecules (Schmidt et al., 2008). Despite of this, only a small proportion of the world's plant species have been investigated for the presence of antifungal compounds, and collection and screening programs are critical due to the high rate of species extinction to avoid the loss of valuable resources.

The genus *Hypericum* includes 500 species distributed all over the world (Nürk et al., 2013a), many of which are used in folk medicine as remedies for wound healing, burns, and gastrointestinal tract inflammation (Silva et al., 2004). The best-known and extensively studied member of this genus is *H. perforatum* L. or Saint John's wort, which is applied for the treatment of mild to moderate depression (Whiskey et al., 2001) and of skin disorders (Schempp et al., 2003). Phytochemical data on *Hypericum* suggest that the genus represents a reliable source for bioactive compounds, which mainly belong to the class of type III polyketide metabolites (Liu et al., 2003). However, little information is available on the antifungal potential of this genus and just a small number of species has been investigated in this regard. Important evidence has been reported by Crockett et al. (2011) and Tocci et al. (2011), who demonstrated that extracts of *H. perforatum* contain compounds active against both plant (*Colletotrichum* spp., *Phomopsis* spp.) and human (*C. albicans*, non-*albicans Candida* species, *Cryptococcus neoformans*) fungal pathogens. Dall'Agnol et al. (2003) and Bridi et al. (2017) also reported antifungal activity of extracts isolated from *Hypericum* species native to Brasil.

Due to high levels of local endemisms that characterize the genus especially in Andean South America (Nürk et al., 2013b), the exploration of new *Hypericum* species as source of pharmaceuticals is of great interest. The peculiar high elevation páramo ecosystem that harbors most species diversity of *Hypericum* in South America is located in the altitudinal range of 3000–5000 m in the northern Andes, forming a tropic-alpine shrub- and grassland ecosystem. The majority of numerous páramo endemic *Hypericum* species are still unexplored mainly due to the difficulties in accessing the rough terrain of their extreme habitats (Crockett et al., 2010). The present study reports for the first time phytochemical profiles and data on antifungal activity of seven *Hypericum* species (*H. cardonae* Cuatrec., *H. carinosum* R. Keller, *H. cuatrecasii* Gleason, *H. garciae* Pierce, *H. humboldtianum* Steud, *H. laricifolium* Juss., and *H. myricariifolium* Hieron.), which are native to the páramo ecosystem in Colombia.

2. Materials and methods

2.1. Plant material

Aerial parts of *H. cardonae*, *H. myricariifolium*, *H. laricifolium*, *H. humboldtianum*, *H. garciae*, *H. carinosum*, and *H. cuatrecasii* have been collected during the year 2012 in the eastern Cordillera of the Colombian Andes. Voucher specimens have been deposited in the herbarium of the Universidad de los Andes (ANDES), Colombia.

2.2. Preparation of crude plant extracts

For each species, 500 mg of dried plant biomass were powdered by grinding and extracted with methanol (drug/solvent ratio = 1:20 w/v) by maceration (3 × 24 h) in the dark. The obtained extracts were evaporated to dryness, weighed and stored at −20 °C until analysis.

2.3. Preparation of H. garciae extracts

Dried aerial parts (1.2 g) of *H. garciae* were powdered by grinding, then serially extracted with chloroform and methanol (drug/solvent ratio = 1:20 w/v). After evaporation of the solvent, the extracts were weighed and stored at −20 °C until analysis.

2.4. Preparation of samples for chemical analysis

Extracts were resuspended in 50% methanol/water and filtered with sterile (0.2 µm) PTFE filters, prior to analysis.

2.5. Chemicals

Methanol and acetonitrile were of LC–MS grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Milli-Q water was used for the chromatography. The majority of the chemical standards are commercially available and were obtained from different suppliers (Vrhovsek et al., 2012). cis-piceid was produced by photochemical isomerization of the trans form, as described by Mattivi et al. (1995).

2.6. LC–MS analysis

Analysis of phenolic metabolites was performed as previously described (Vrhovsek et al., 2012) with few modifications. A Waters Acquity UPLC system (Milford, MA) consisting of a binary pump, an online vacuum degasser, an autosampler and a column compartment was used. Phenolic compounds were separated at a temperature of 40 °C on a Waters Acquity HSS T3 column 1.8 µm, 150 mm × 2.1 mm (Milford, MA, USA). The mobile phase was composed of component A (0.1% formic acid in water) and component B (0.1% formic acid in acetonitrile). The flow was set to 0.4 ml/min, and the gradient profile was: 0 min, 5% B; from 0 to 3 min, linear gradient to 20% B; from 3 to 4.3 min, isocratic 20% B; from 4.3 to 9 min, linear gradient to 45% B; from 9 to 11 min, linear gradient to 100% B; from 11 to 13 min, wash at 100% B; from 13.01 to 15 min, the re-equilibrated to the initial conditions of 5% B. The injection volume was 2 µl for both sample and standard solutions. Each sample was analyzed in triplicate. After each injection, the needle was rinsed with 600 µl of a weak washing solution (water/methanol, 90:10) and 200 µl of a strong washing solution (methanol/water, 90:10). Samples were kept at 6 °C during the analysis.

Mass spectrometry detection was performed on a Waters Xevo TQMS (Milford, MA, USA) instrument equipped with an electrospray (ESI) source. Capillary voltage was 3.5 kV in positive mode and −2.5 kV in negative mode; the source was kept at 150 °C; the desolvation temperature was 500 °C; cone gas flow, 50 l/h; and desolvation gas flow, 800 l/h. Further MS parameters are reported in Vrhovsek et al. (2012).

Quantification was done using Waters MassLynx 4.1 and TargetLynx software. All compounds were quantified via external calibrations.

2.7. Microorganisms and media

For antifungal susceptibility testing, strains from the American Type Culture Collection (ATCC, Rockville, MD, USA; *S. cerevisiae* ATCC4040002 and *C. albicans* ATCC MYA-2876) were used as references. The environmental strain *S. cerevisiae* BB1533, and human gut clinical isolates, *S.cerevisiae* YUC5, *C. albicans* YN5, *C. albicans* YN7, *C. albicans* YL1, *C. parapsilosis* YB1 (Di Paola et al., unpublished) *C. lusitaniae* YHS217, *C. lusitaniae* YHS72 (Strati et al., 2016), *C. glabrata* MFB004, *C. intermedia* MFB022-1, *C. intermedia* AD125, *C. tropicalis* MFB035-1, and *C. tropicalis* RTT037-3 (Strati et al., unpublished) were tested for their susceptibility against the applied drugs.

Yeasts were grown on Sabouraud agarized medium for 48 h at 30 °C and resuspended in distilled water to a concentration of 1–5 × 10^5 CFU/ml before testing.

2.8. Antifungal susceptibility testings

Yeasts were tested for their susceptibility to *Hypericum* spp. extracts (8 dilution series, ranging from 1000 µg/ml to 8 µg/ml) and to *H. garciae* extracts (7 dilution series ranging from 125 µg/ml to 2 µg/ml),