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Hypericum species in the Páramos of Central and South America: a special focus upon *H. irazuense* Kuntze ex N. Robson

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Abstract

Knowledge about members of the flowering plant family Clusiaceae occurring in the tropical mountain regions of the world is limited, in part due to endemism and restricted distributions. High altitude vegetation habitats (Páramos) in Central and South America are home to numerous native *Hypericum* species. Information related to the phytochemistry of páramo *Hypericum*, as well as ecological factors with the potential to influence chemical defenses in these plants, is briefly reviewed. Results of the phytochemical analysis of *Hypericum irazuense*, a species collected in the páramo of the Cordillera de Talamanca in Costa Rica, are presented. Lastly, guidelines for the viable and sustainable collections of plant material, to facilitate future investigations of these interesting plants, are given.

Keywords

Clusiaceae; *Hypericum*; Montane; Páramo; Phytochemistry; Xanthone

Introduction

Species of *Hypericum* (Clusiaceae) are primarily temperate in distribution, but can also be found in tropical regions of the world at high altitudes, particularly in South America and Africa. The American Cordillera is a nearly unbroken series of mountain ranges extending from the Alaskan Range to the Andes, which stretch to the southern tip of South America. The highest peaks are found in the Cordillera de Talamanca in Costa Rica and adjacent Panama in Central America and in the Andes Mountains of Columbia, Ecuador, Venezuela, Peru and Bolivia in South America. In the altitudinal range of 3,000–5,000 m in these

countries, above the timberline but below the snow-line, a specific vegetation type called the páramo is found, characterized by huge Asteraceae forming rosettes (such as *Espeletia*) and cushions (*Werneria*), bunchgrasses (*Calamagrostis*) and scattered evergreen shrubs (including *Hypericum*) (Luteyn 1999). Knowledge about *Hypericum* species occurring in these regions is still scattered and sparse, in part due to the high proportion of endemism and the resulting restricted distribution patterns.

The 457 species of *Hypericum* occurring world-wide have been divided into 36 taxonomic sections on the basis of morphological characters (Robson 2003). The most well-known of these species is the common St. John's Wort (*H. perforatum* L.), which has a long history of medicinal use for various purposes, and of which extracts are currently sold in Europe and North America to treat mild to moderate depression. Medicinally active constituents include flavonoid glycosides, naphthodianthrone (e.g. hypericin and pseudohypericin) and acylphloroglucinol derivatives (e.g. hyperforin) (Müller 2005). The presence of *H. perforatum* at lower elevations throughout drier habitats in Central and South America is a result of recent introduction and escape from cultivation (Blumenthal et al. 2000). The majority of *Hypericum* species native to high mountain regions of Central and South America belong to the taxonomic section *Brathys* and to a lesser extent, *Trigynobrachys*. On the basis of earlier morphological studies, basal species of sect. *Brathys* have been considered most closely related to species in sect. *Campyloporus* found in the mountains of East Africa in similar habitat, which on this continent is described with the terms "afroalpine" or "moorland" (Robson 1987; Luteyn 1999).

The general working hypothesis that plants growing under extreme conditions possess unique biochemical adaptations and thus interesting phytochemistry has been thoroughly tested, particularly in desert environments. Due to the energetic "cost" to the plant of replacing vegetative or reproductive tissue lost to herbivory, disease or parasitism, plants growing in these environments often produce a diverse range of secondary metabolites as defense compounds (Timmermann 1999). Because the presence and amount of these compounds can contribute to the survival of the plant and its progeny, evidence suggests that individuals possessing the ability to biosynthesize defensive secondary metabolites enjoy a selective advantage over others lacking this ability (Harborne 1993). Plants growing in the páramo are subjected to numerous environmental stress factors, including high levels of UV radiation; low amounts of organic nutrients, certain minerals and available water in soils; rapid temperature changes; and, recently, anthropogenic factors (e.g. cattle herbivory), all of which have the potential to influence chemical constituent production.

This information indicates that *Hypericum* species occurring in this environment would be interesting targets of phytochemical research. In this review, a brief glimpse into ecological factors operating in the system is given in order to understand more about forces that are responsible, at least in part, for the selection and maintenance of secondary metabolite pathways in páramo *Hypericum* species. A review of what is known about the chemistry of *Hypericum* species native to the páramo regions of Central and South America is provided and results of the phytochemical analysis of *Hypericum irazuense*, collected in the páramo of the Cordillera de Talamanca in Costa Rica, are presented. Finally, suggestions are offered for ways in which viable and sustainable collections of material may be made to facilitate future phytochemical investigations of these interesting plants.

Ecology of páramo *Hypericum*s

In the northern-central Andes, a complex mixture of habitats occurs within the páramo including dry to moist grasslands (either pure or intermixed with low shrubs), acidic bogs, peat-lands and rocky outcrops. Many *Hypericum* species occur in these habitats, often with

distinctly localized distributions (see Table 1). *Hypericum strictum*, *H. caracasenum*, *H. stenopetalum*, *H. lancioides* and *H. juniperinum* (sect. *Brathys*), for example, are species growing in tall grasslands interspersed with shrubs (1–5 m tall) on moderately well-drained soils in the mountains of Ecuador, Columbia, Peru and Venezuela. In the Eastern Cordillera, the Sierra Nevada del Cocuy and the Páramo de Sumapaz represent two of the most diverse biogeographic centres in the region, in which a number of endemic species including *H. prostratum*, *H. cymobrachys* and *H. papillosum* are found. At slightly lower elevations in parts of Bolivia and Peru, *H. andinum* occurs in a habitat characterized by mixed grasslands with varying proportions of trees and low shrubs, which has often been subjected to impact by livestock, agriculture and fire (NatureServe 2009).

In the Eastern Cordillera of northern Ecuador, *H. silenoides* (sect. *Trigynobrachys*) and *H. aciculare* (sect. *Brathys*) prefer moister conditions, occurring on humid slopes also inhabited by bamboo (*Neurolepis aristata*), large rosette-forming plants (e.g. *Puya*), and ferns (*Jamesonia goudotii*), as well as in flat cushion bogs in the tussock grass páramo, associated with *Oreobolus cleefii* (Cyperaceae) and *Xyris subulata* (Xyridaceae). The preference of particular species of *Hypericum* for certain soil types and conditions has been documented, and in the páramo many species seem to favor acidic soils, particularly those overlying volcanic bedrock. Such species as *H. decandrum* and *H. thuyoides* (sect. *Brathys*) frequently occur in the tussock grass communities found on dormant volcanoes in the central Andes (Olivera and Cleef 2009). *Hypericum juniperinum*, *H. phellos* and *H. mexicanum* (sect. *Brathys*), for example, have been listed as abundant in the Vereda Alto Grande Pamplona volcanic reserve in Columbia (Sánchez et al. 2007).

Hypericum is an integral part of the páramo throughout Central and South America. In fact, *Hypericum* is considered such a standard element of this vegetation type that the Botanical Garden in Bogotá, Columbia, has planted several species in their demonstration garden, which displays the representative flora of the region (Torres 2003). *Hypericum laricifolium* (sect. *Brathys*), one of the more abundant and widely-distributed species, is diagnostic for areas of shrubland that have succeeded former cropland carved out of successional moist montane forests (often called ‘elfin’ or ‘cloud’ forests) by rural populations living in the Andes of Ecuador and Columbia. It grows on plains and gentle slopes of the mountains, on moist to moderately drained soils (NatureServe 2009). This species has been frequently observed in areas that have been repeatedly burnt or disturbed (e.g. by cultivation), and seems to be able to slowly recover, even to become one of the dominant species of the successional vegetation complex (Rabey 1993; Olivera and Cleef 2009).

In contrast to *H. laricifolium*, which appears to recover from various anthropogenic impacts relatively quickly, many *Hypericum* species native to the páramo lack this flexibility. In a study by Jaimes and Sarmiento (2002), the regeneration of páramo vegetation in an area of the Eastern Cordillera Oriental in the Colombian Andes was observed for a period of 17 years following agricultural disturbance (i.e. the planting of a potato crop). In the period of 4–7 years after the disturbance, plants of *H. juniperinum* (local name: *chite* or *escobo*), *H. mexicanum* (*chite pegajoso*) and *H. goyanesii* (*guardarrocío* or *pinito de páramo*) (sect. *Brathys*) slowly established, but the availability of “nurse” shrubs in the area, which provide microhabitats with higher amounts of soil-bound water and nutrients and buffer wind and temperature stresses, greatly influenced the survival rate of the seedlings. *Hypericum strictum* (*chite*) was observed only in the recuperating páramo zone a minimum of 15 years after the disturbance had taken place and required 13–16 years to re-establish a balance with associated vegetation. After an average of 15 years, the colonizing species of the fallow zones were slowly replaced by the more gradually recovering native species of the páramo, although their frequency and density did not regain pre-disturbance levels.

Extremely slow re-sprouting rates have been recorded for *H. caracasenum* and *H. irazuense* occurring in regions of the páramo in Costa Rica damaged by fire, with the observations that entirely bare ground (i.e. lacking residual nurse shrubs) was still not re-colonized 3 years after the fire (Janzen 1973; Gannon and Willig 1996). In general, páramo *Hypericum* species with restricted distributions or distinct habitat requirements show slower regeneration after fire, as is also the case with *H. lancioides* (Verweij and Budde 1992). As an interesting addendum, the effects of disturbance on *Hypericum* are not always negative, particularly in zones where water and nutrients are plentiful and where some ground cover remains to shelter establishing seedlings. For example, *H. juniperinum* has been observed in both the undisturbed soil flanking mining sites and colonizing disturbed mine tailings in a subpáramo zone near Bogotá, Columbia. On the mine tailings, the plants appeared healthy and displayed high regeneration rates, possible due to higher light availability. In contrast, in the moist flanking semi-forested areas, growth was slower and plants were overall smaller and less frequent due to competition with co-occurring shrubs and trees (Arias-Escobar and Barrera-Cataño 2007).

The opportunistic tendency of particular *Hypericum* species to colonize disturbed ground suggests interesting future research directions. Although these varied findings may initially appear only to be of significant interest to the plant ecologist, the enterprising phytochemist desiring to collect plants in the páramo would also be advised to pay careful attention to the implications of these studies. Such reports not only serve as good initial guides for the distribution of particular species, but often contain critical information regarding their conservation status, habitat preferences and, in studies of the particular species' response to disturbance, information that allows the phytochemist to assess the potential impact of collecting plant material.

Economic use of páramo *Hypericum* species

In the Ecuadorian Andes, the use of the páramo dates from pre-Incan times, as evidenced by the presence of fortifications, strategic outlooks and reservoirs in several locations. As a result of the Spanish colonization in the sixteenth century, new elements such as camels and horses (used for transport across the páramo), and larger herds of livestock (cows, bred for labor and food and sheep, bred for food and wool) were introduced to the páramo ecosystem, each with a unique environmental impact. These impacts differed significantly from those that had been previously imposed through traditional uses of the páramo by the smaller populations of indigenous cultures, including grazing of small herds of livestock, cultivation of Andean-adapted tuber crops at lower elevations (primarily subpáramo) and breeding of native animals such as the guinea pig.

Other recent changes that have affected the páramo vegetation include repeated burning cycles to induce the growth of young seedlings (supposedly more palatable to livestock); establishment of non-native timber trees (e.g. *Pinus radiata*); intensification of agriculture (resulting in contamination of soil and water with agrochemicals); mining; livestock (resulting in overgrazing, soil compaction, water pollution), and erection of structures (new human settlements due to population growth, communication systems equipment, military bases) (de la Torre et al. 2008). A specific example in the latter category is the erection of numerous cell towers to support telecommunications during the past decade, resulting in the construction of access roads and bulldozing and/or clearcutting of the tower sites. Prior to the construction of such towers, an environmental impact report must be submitted to the government, including information about the potential impact on local flora. Several examples of such environmental impact reports listing páramo *Hypericum* species among the "affected flora" were found during this literature review (for a recent example, see ENTRIX 2009).

Again, however, some species of *Hypericum* in the páramo seem to be able to adapt to and even take advantage of disturbances in their habitats, so long as an available seed bank is maintained. The increased presence of certain species is, in fact, often suggestive of human activity in the páramo. *Hypericum aciculare* and *H. mexicanum*, for example, are listed in the Cordillera Oriental in Columbia as indicator species for biodegraded or polluted soils resulting from compaction due to overgrazing by livestock, surface erosion, contamination by agrochemicals, and repeated burning (Salamanca and Camago 2000). Although the grasslands between 3,500 and 4,000 m in the Venezuelan Andes are subjected to extensive grazing by livestock during the rainy period of March–October, ecologists have observed that shrubby species of *Hypericum* growing in the páramo are generally not subjected to herbivory. In the wet moorlands, plants such as *H. laricifolium* and *H. lancioides* are considered by locals as fair to poor forage material for the animals (Molinillo and Monasterio 2002). Whether this is due to the availability of more palatable plants or due to a specific chemical defense on the part of the *Hypericum* species remains to be investigated.

Which plants are used by indigenous cultures in Andean countries has not yet been thoroughly studied and these plants, including medicinal species, are primarily wildcrafted. Several *Hypericum* species are collected from the páramo, particularly in Ecuador, Peru and Columbia, for sale in local markets. *Hypericum laricifolium*, which is often locally abundant and can attain a robust size, serves as a source of firewood and a dye plant (a decoction of the leaves and flowers gives a yellow to greenish tint to wool and cotton) (Vásquez and Hofstede 2006; de la Torre et al. 2008). An interesting project conducted by the INIAP-DENAREF (Instituto Nacional Autónomo de Investigaciones Agropecuarias-Departamento Nacional de Recursos Fitogenéticos y Biotecnología) in Ecuador is to develop molecular tools including RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism) and SSR (simple sequence repeat) markers to identify samples of *H. laricifolium* from local markets, presumably due to problems with adulteration (Roca 2003). Such techniques have been utilized successfully to distinguish and identify other species of *Hypericum* (Teuchen et al. 2004). Until recently, such plants have had restricted markets, a situation that could be drastically altered by the effects of globalization. When considering the potential of a particular medicinal plant for local cultivation, for the purposes of sale and exportation on the global market, one must consider that the biomass productivity in these regions is limited by light and water availability and soil quality, which in turn are significantly affected by anthropogenic factors, particularly fire and livestock (de la Torre et al. 2008).

Already in the early nineteenth century certain páramo *Hypericum* species, including *H. thuyoides*, *H. aciculare* and *H. humboldtianum*, seeds or plants of which were originally collected in Ecuador and Columbia and survived the long journey back to Europe, were valued as unique ornamentals by German shrubby plant enthusiasts (Dietrich 1835). More recently in the region of Boyacá in Columbia, local peoples have supplemented their income through the sale of *H. lancifolium* Gleason (cited as “*H. lancifolius*”, local name *velilla*) as an ornamental plant (Corpocaldas/Conservación Internacional 2007). The potential for various native *Hypericum* species to be used as foliage stock in ornamental bouquets has been explored in Columbia, and although certain species possess desirable characteristics such as lengthy, slender, non-brittle stems, an attractive silhouette, dense foliage, and shiny leaves that do not quickly drop, development of these species for commercial trade has not been pursued (Gutiérrez et al. 2007). *Hypericum juniperinum* and *H. laricifolium* may be of potential value as native landscape plants, due to their noted tolerance of disturbed ground and the utilization of their flower buds, fruits and leaves as food by birds in Columbia and Ecuador (de la Torre et al. 2008).

Phytochemical studies of páramo *Hypericum*

Although relatively few species of *Hypericum* native to the páramo have been phytochemically examined, several studies of species belonging to sections *Brathys* and *Trigynobrathys* occurring at lower elevations in Central and South America have been published. These include reports of the presence of anthraquinone derivatives and flavonoids in *H. strictum* (sect. *Brathys*) (Makovetska 2001); of flavonoids, xanthenes, phloroglucinol derivatives and triterpenic acids in *Hypericum brasiliense* Choisy (sect. *Trigynobrathys*) (Abreu et al. 2004); and of phloroglucinol derivatives in *H. polyanthemum* Kotsch ex Reichardt (sect. *Trigynobrathys*) (Bernardi et al. 2008). Abreu et al. (2004) noted that the phloroglucinol derivative (detected only in the roots) and flavonoid glycosides (detected mainly in the shoots) were present in the highest amounts during the flowering stage, while xanthenes (found in all plant parts) and triterpenic acids (mainly in the shoots) were highest during fruiting.

In the work of Bernardi et al. (2008), the highest levels of phenolics were isolated from vegetative tissue after 4 months of growth in the field and fully open flowers (as opposed to buds or withering flowers) possessed the highest amounts of all compounds. Although the results from these individual species can not be directly extrapolated to related species of sections *Brathys* and *Trigynobrathys* occurring in the páramo, such studies support the hypothesis that they possess biosynthetic pathways to create bioactive secondary metabolites and that the amounts present are influenced by environmental factors. These studies also clearly demonstrate that a collection strategy must be carefully formulated to target specific compound classes.

Among species found in the páramo, *H. silenoides* (sect. *Trigynobrathys*) tested negative for alkaloids, a non-surprising result since alkaloids have not been detected from any species of *Hypericum* to date, and in fact, nitrogen-containing compounds have been only rarely reported (Sáenz and Nassar 1965). A phytochemical investigation of cell cultures of *H. gnidioides* (sect. *Brathys*) revealed the presence of several xanthenes (Fig. 1, **1–5**) (Abd El-Mawla 2005). Two separate researchers tested for the presence of hyperforin (a phloroglucinol derivative present in *H. perforatum*) in *H. gnidioides*, with negative results (Klingauf 2004; Boubakir 2006).

In a study of the phytochemical constituents of the leaves of *H. laricifolium*, collected in the páramo in Venezuela, two xanthenes were identified (Fig. 1, **1** and **6**) (Ramírez et al. 2004). Material from this species has also been collected in the páramo in Ecuador (Pichincha) and phytochemically investigated, yielding an array of secondary metabolites including two caffeic acid esters of long-chain aliphatic alcohols (Fig. 1, **7–8**) obtained as a mixture, sterols (**9–10**), triterpenoids (**11**), benzoic and cinnamic acid derivatives (**12–16**), flavonols and flavonol glycosides (**17–21**). These were tested in vitro for anti-inflammatory activity, measured in terms of inhibition of two forms of the cyclooxygenase enzyme (COX-1 and COX-2). Most inhibited COX-1 and -2 by less than 30% and were considered inactive, however, quercetin (**17**) inhibited COX-1 by $44 \pm 2\%$ (concentration: 200 μM) and the mixture of caffeic acid esters (**7–8**) inhibited COX-1 by $52 \pm 2\%$ (concentration: 1000 μM). IC_{50} values for these compounds were not obtained due to solubility problems. Caffeic acid (**12**) additionally inhibited COX-2 by $32 \pm 16\%$ (concentration: 100 μM) in a non-dose-dependent manner (El-Seedi et al. 2003). An ethanolic extract of leaves of *H. laricifolium*, collected in the páramo in Peru (Amazonas), displayed a weak MIC of 250 $\mu\text{g/ml}$ against *Candida albicans* ATCC 10231, but specific bioactive constituents were not identified (Achata 2005).

Hypericum laricifolium is morphologically highly variable, but this variability appears to be continuous throughout its distribution. In northern Ecuador and adjacent Columbia, the leaves are relatively broad (ca. 6–8 mm), while moving northeastward towards Venezuela and southward toward Peru, a trend towards narrower (to ca. 2–3 mm) leaves emerges. At the extreme northeastern end of its distribution, the plant has been called “*H. laricoides*” and this name appears in some publications (Robson 1987). It is not surprising, therefore, that the phytochemical studies cited above resulted in the isolation of different compounds. A phytochemical study of *H. laricifolium* plants collected along a geographic gradient in the Cordillera Central and Oriental from Venezuela to Peru would be a worthy project.

Phytochemical investigation of *Hypericum irazuense* Kuntze ex N. Robson

Background

Hypericum irazuense is a perennial shrub or small tree that can reach up to 5 m in height. The imbricate leaves are narrowly elliptical and lightly appressed along the stem. The flowers attain a diameter of nearly 3 cm under favorable conditions and are a rich buttery yellow color (Fig. 2). When crushed, the foliage gives off a strong pine-scent. The species is distributed on slopes in the open páramo above 2,500 m, often associated with clumps of bamboo (*Chusquea*), along the American Cordillera from Costa Rica (San José, Cartago, Limón) into Panama (Chiriquí) (Robson 1987, and pers. obs.) (Fig. 3). In Costa Rica, the flowers are pollinated primarily by bumble bees (e.g. *Bombus ephippiatus* Say) (Gargiullo 2008). Beetles of the genus *Lupraea* (Coleoptera: Chrysomelidae) have been observed feeding upon the leaves (Flowers and Janzen 1997). Plants of *H. irazuense* living in the páramo of the Cordillera de Talamanca in Costa Rica experience such stresses as extreme temperature changes, high levels of UV radiation, water and nutrient limitations and, sometimes, anthropogenic impacts (e.g. fire, livestock) (Farji-Brener et al. 2009).

As part of our continuing investigations of the phytochemistry of the genus, and to improve our knowledge specifically of the phytochemistry of páramo *Hypericum* species, an investigation of *H. irazuense* was conducted.

Experimental

General procedures—TLC precoated silica gel 60 F_{254} (Merck); detection by spraying with vanillin/H₂SO₄, followed by heating. Vacuum liquid chromatography (VLC): silica gel 60 (150 g, particle size 0.040–0.063 mm, Merck). Solid phase extraction: Isolute SPE cartridges (C18, 2 g packing with 6 ml volume, Biotage), elution with H₂O/MeOH mixtures (see “Extraction and isolation”). Gas chromatography–mass spectrometry (GC–MS): Agilent 7890A GC-system equipped with a 7683B series injector and 5975C VL_{MSD} (Agilent Technologies, USA) using an HP-5MS 5% phenyl methyl silox column (30 m × 250 μ m × 0.25 μ m film thickness, Agilent) and helium as carrier gas. Temperature program (45°C for 2 min, increasing 4°C/min to 250°C, held 2 min, for essential oil components and 45°C for 2 min, increasing 6°C/min to 300°C, held 20 min, for sterols). Injections were applied split (ratio, 80:1 and 50:1) with a port temperature of 240 or 280°C. MS were recorded at 70 eV. Identification of components was carried out through comparison with data published in Adams (1995) as well as by using the commercially-available NIST 05 and Wiley Mass Spectral Libraries. High performance liquid chromatography (HPLC): Analytical—Agilent 1100 Separations Module (Agilent Technologies, USA) using a Zorbax SB-C18 column (3.5 μ m, 2.1 × 150 mm; Agilent), coupled to a UV detector with a flow rate of 300 μ l/min (gradient, see “Extraction and isolation”); preparative—Varian R PrepStar Model SD-1 with a Dynamax R Solvent Delivery System Model SD-1 (Varian Inc., USA) using a LichroSpher 100-C18 column (10 μ m, 25 × 250 mm, Varian), coupled to a Dynamax R Absorbance Detector Model UV-1 (detection at 210 and 254 nm) with a flow

rate of 20 ml/min (gradient, see “Extraction and isolation”). HPLC–DAD/ESI–MS (pos.) was performed on a Thermo Finnigan Surveyor liquid chromatography instrument with Thermo Quest Surveyor photodiode array detector, autosampler, and MS pump, and a Thermo Finnigan LCQ-XP mass detector equipped with an electrospray ionization (ESI) source run by Xcaliber software (Thermo Fisher Scientific, USA). Mass spectra were detected and recorded in a scan range of m/z 50–1,000, using a transfer capillary temperature of 350°C, a spray voltage of 5.00 kV and a sheath gas flow of 70 units. NMR Spectra: ^1H -, ^{13}C -, and 2D-NMR experiments (HSQC, HMBC, DQF-COSY) were performed on Varian UnityInova-400 and -600 MHz spectrometers; experimental parameters according to Seebacher et al. (2003); δ in ppm, J in Hz. Compounds were dissolved in either chloroform- d_1 or pyridine- d_5 , with tetramethylsilane (TMS) as an internal standard.

Plant material—*Hypericum irazuense* material, consisting of leaves, stems and flowers from the upper 30 cm of the branches was collected by Dr. Sara Crockett and Dr. Wolfgang Schühly in March 2007 in the Cordillera de Talamanca (Costa Rica), above the city of Villa Mills, ca. 1 km E of the Pan-American highway in the páramo vegetation at 3,100 m elevation. A voucher specimen was deposited with InBio (Intituto Nacional de Biodiversidad) in San José, Costa Rica. Collection and exportation of plant material was granted through permit number 01490 issued by SINAC-MINEA (El Sistema Nacional de Areas de Conservación del Ministerio del Ambiente y Energia) as part of an ongoing project entitled “Investigación biológica y botánica de un grupo selecto de plantas de Costa Rica.”

Extraction and isolation—Plant material was air-dried at ambient temperature. The dried, ground material (500 g) was extracted (at room temperature) with MeOH (4 $\times$, 1.5 l). The combined MeOH extracts were evaporated in vacuo to give ca. 100 ml of concentrated extract, which was mixed with 900 ml of distilled H₂O and partitioned against DCM (6 $\times$, 500 ml). Combined DCM partitions were concentrated *in vacuo* to give ca. 17.5 g extract. The aqueous MeOH phase was subsequently partitioned against n-BuOH (3 $\times$, 500 ml). These combined partitions were mixed 1:1 with water and concentrated in vacuo to give ca. 13 g extract. Due to interesting TLC characteristics, the DCM extract was selected for further analysis.

The DCM extract (17.5 g) was subjected to VLC using a gradient elution by hexane:EtOAc followed by EtOAc:MeOH. Earlier eluting fractions contained highly non-polar substances that were tentatively identified from NMR spectra and GC–MS analysis as α -trans-bergamotene (96% certainty), cembene (99%), α -pinene (94%), β -pinene (96%) and limonene (95%). α - and β -pinene were identified as particularly enriched in these fractions. Increasing the polarity of the eluent yielded fractions containing a triterpenic acid and 5 xanthenes (Fig. 4, **HI-1** to **HI-6**). Compound **HI-1** was eluted with 90:10 hexane:EtOAc as a mixture with compound **HI-2**. This mixture (66 mg) was dissolved in 2.2 ml MeOH and chromatographed over a C18 SPE column, conditioned with MeOH, and eluted with a MeOH-DCM gradient. Indicative ^{13}C -NMR shifts allowed the identification of **HI-1** from early eluting fractions (yield: 36 mg. ca. 90% pure) through comparison with values from literature (Schühly et al. 1999) and compound **HI-2**, which formed a white precipitate upon drying, from subsequent fractions (yield: 22 mg). **HI-2** was identified by comparison of ^1H - and ^{13}C -NMR and mass values with those cited in Rocha et al. (1994). Compounds **HI-3** and **HI-4** were isolated from the VLC fraction eluted with 83:17 hexane:EtOAc. This dark green fraction (418 mg) was divided into 2 portions, each dissolved in 1 ml MeOH and chromatographed over a C18 SPE column, conditioned and eluted with MeOH. Fractions were combined on the basis of similarity, assessed using TLC and analytical HPLC. Preparative HPLC was then used to purify compounds from the resulting extract (378 mg),

using a gradient of 75:25 ACN:H₂O to 100% ACN over 25 min. **HI-3** (13 mg) and **HI-4** (50 mg) were obtained through this process, and identified by comparison of their ¹H- and ¹³C-NMR and mass values with those cited in literature (Kobayashi et al. 1997 for **HI-3**; Botta et al. 1986 for **HI-4**). Compounds **HI-5** and **HI-6** were isolated from the VLC fraction eluted with 80:20 hexane:EtOAc. This dark green fraction (269 mg) was dissolved in 1 ml MeOH and chromatographed over a C18 SPE column, conditioned and eluted with MeOH. The first eluted fraction (227 mg) was further fractionated using preparative HPLC, eluted with isocratic 60:40 ACN:H₂O for 10 min and then a gradient to 100% ACN to 18 min, yielding compounds **HI-5** (16 mg) and **HI-6** (63 mg). These substances were identified by comparison of their ¹H- and ¹³C-NMR and mass values with those cited in literature (Botta et al. 1986 for **HI-5**; Chang et al. 1989 for **HI-6**).

Discussion

All isolated compounds were identified by their spectral properties and by comparison of measured spectral and mass values from those of published literature. Betulinic acid (**HI-1**) is a secondary metabolite that is present in many species of flowering plants, and although it has not been previously reported from a member of *Hypericum* sect. *Brathys*, it has been isolated from *H. japonicum* Thunb. ex Murray (Fu et al. 2004) and *H. brasiliense* (Rocha et al. 1994) (both sect. *Trigynobrathys*). In addition, the 3-epimer of betulinic acid has been reported from *H. laricifolium* (sect. *Brathys*) (El-Seedi et al. 2003).

Among the isolated xanthenes, **HI-2** [1,5-dihydroxyxanthone] has been previously isolated from the stems and roots of *H. brasiliense* and displayed an antimycotic activity against *Cladosporium cucumarinum* (Rocha et al. 1994). This substance has also been previously identified from the inner bark of *Mesua ferrea* L. (Clusiaceae) (Chow and Quon 1968). Compounds **HI-3** through **HI-6** are here reported for the first time from *Hypericum*. **HI-3** [4-(1,1-dimethyl-2-propenyl)-1,5,6-trihydroxy-3-methoxy-xanthen-9-one or “Isocudranixanthone B”] has been previously isolated from the roots of *Maclura cochinchinensis* (Kobayashi et al. 1997) and from the root bark of *Cudrania tricuspidata* (both Moraceae) (Lee et al. 2005). In the latter study, **HI-3** displayed good radical scavenging activity against DPPH (IC₅₀ = 31.8 μM) and moderate cytotoxic activity (LD₅₀ ranging from 71.3 to 43.9 μM) against the human cancer cell lines HT-29 (colon), HL-60 (promyelocytic leukemia), SK-OV3 (ovary), AGS (stomach) and A549 (lung). **HI-4** [5,9,10-trihydroxy-2,2-dimethyl-12-(3-methylbut-2-enyl)-pyrano-[3,2-*b*]-xanthen-9-one] and **HI-5** [1,3,5,6-tetrahydroxy-2,4-bis(3-methylbut-2-enyl)-xanthen-9-one] have been previously isolated from other members of the Clusiaceae, including from the root bark of *Vismia guineensis* (L.f.) Choisy (Botta et al. 1986) and the stem bark of *Garcinia lancilimba* C. Y. Wu ex Y. H. Li (Yang et al. 2007). **HI-4** and **HI-6** [4-(1,1-dimethyl-2-propenyl)-1,3,5,6-tetrahydroxy-2-(3-methylbut-2-enyl)-xanthen-9-one or “Gerontoxanthone I”] have additionally been isolated from the roots of *Cratoxylum formosum* (Jack) Dyer (Boonsri et al. 2006), and **HI-6** has been found in the bark of *Cudrania cochinchinensis* (Moraceae) (Chang et al. 1989). The latter substance displayed good activity (MICs = 3.1–6.2 μg/ml) against a range of vancomycin-sensitive and vancomycin-resistant enterococci (*Enterococcus faecalis*, *E. faecium* and *E. gallinarum*), as compared to the activity (MIC = 0.78–1.56 μg/ml) of the positive control, linezolid (Fukai et al. 2005).

This series of isolated xanthenes from *H. irazuense* illustrates the diversity of secondary metabolites that can be produced through biosynthetic elaboration upon a simple base structure (in this example, **HI-2**). 1,3,5-trihydroxyxanthenes have been shown to give rise to 1,3,5,6-tetrahydroxyxanthenes via 6-hydroxylation (Schmidt et al. 2000), and the presence of an oxygen at positions 1,3,5 and 6 of the isolated compounds **HI-3** through **HI-6** is seen (Fig. 4). In compounds **HI-3**, **HI-5** and **HI-6**, further modifications to the base structure in

the form of prenylations and in **HI-3**, methylations, have taken place. **HI-4** is very closely related to **HI-5**, differing only in that a cyclization has taken place. It is interesting that in the case of **HI-4** and **HI-5**, the prenyl group has been attached via the C-1 group of the prenyl donor (dimethylallyl diphosphate or DMAPP), whereas in the case of **HI-3** and **HI-6**, the prenyl has been attached via the C-3 group of the donor (i.e. reverse prenylation). Such findings stimulate the development of hypotheses to be tested during the elucidation of underlying biosynthetic pathways (see Fig. 5).

Bioactivity testing of xanthenes isolated from *H. irazuense*, as well as phytochemical investigations of the related Costa Rican páramo species *H. costaricense* (sect. *Brathys*) and *H. thesiifolium* (sect. *Trigynobrathys*) are ongoing in our laboratories.

Final remarks

A review of information related to the phytochemistry and, in part, the ecological requirements of páramo *Hypericum* species has revealed several issues relevant to phytochemists wishing to conduct future investigations in these regions. Proposed research must consider the unique characteristics of each species, particularly taking into account their formal conservation status in the country of origin, sensitivity to environmental disturbance, slow regeneration of vegetative and flowering material upon trimming or wounding, and often low levels of regeneration from seed. During the formulation stage of a collection strategy, the researcher should consider which collection season would yield optimum results; what part(s) of the plant should be collected to target specific classes of secondary metabolites; and whether the possibility exists to cultivate plants from collected seed and/or shoots for analysis (perhaps in collaboration with a local botanical garden).

Examples of such efforts being made in Columbia for *H. goyanesii* (Manrique and Montes 2001) and *H. humboldtianum* (Manrique and Granada 2003), in order to conserve the genetic diversity inherent in particular accessions by using vegetative in vitro propagation techniques, indicate that such techniques could be applied to other endemic species of páramo *Hypericum*. The work by Abd El-Mawla (2005) has demonstrated that, using the highly sensitive analytical tools available today (e.g. HPLC coupled to MS or NMR), studies may be performed using relatively small amounts of plant material. Should highly interesting bioactive compounds be discovered in these species, a scale-up of such research would require further investigation into the propagation of plants to provide a sustainable source of material. During our work with various *Hypericum* species during the past years, we have observed that little extra effort is required to collect either young shoots or seeds (depending on season) during collection of material for phytochemical investigation, and subsequently to root and/or germinate this material (Crockett, pers. obs.; Dirr et al. 1999). Future projects involving phytochemical investigations of páramo *Hypericum* species, particularly those with restricted distributional ranges, may be pursued most efficiently and effectively through strong collaborations between phytochemists, botanists and ecologists working in the region.

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Fig. 1.
Secondary metabolites reported from páramo *Hypericum* species

Fig. 2.
Hypericum irazuense in flower. Photo credit: S. Crockett

Fig. 3. Habitat of *Hypericum irazuense* (background) and *H. costaricense* (foreground) in the Cordillera de Talamanca, Costa Rica. Photo credit: S. Crockett

Fig. 4.
Compounds isolated from *H. irazuense*

Fig. 5.
Hypothesized biosynthetic relationships among xanthenes isolated from *H. irazuense*

Table 1

Species of *Hypericum* reported to occur in Páramo habitat

Section	Species ^a	Species authority	Distribution ^b
Brathys	<i>H. aciculare</i>	Kunth	EC (Azuay, Loja) PE (Piura, Amazonas)
Brathys	<i>H. acostanum</i>	N. Robson	EC (Loja, Morona-Santiago)
Brathys	<i>H. andinum</i>	Gleason	PE (La Libertad, Junin, Huancavelica, Cuzco, Puño) BO (La Paz, Cochabamba, Santa Cruz)
Brathys	<i>H. asplundii</i>	N. Robson	EC (Pichincha)
Brathys	<i>H. baccharoides</i>	Cuatrec.	CO (Magdalena) VE (Zulia)
Brathys	<i>H. bryoides</i>	Gleason	CO (Norte de Santander, Santander, Boyacá)
Brathys	<i>H. callacallanum</i>	N. Robson	PE (Amazonas)
Brathys	<i>H. caracasana</i> ssp. <i>caracasana</i>	Willd.	VE (Distrito Federal, Miranda, Aragua, Anzoátegui)
Brathys	<i>H. caracasana</i> ssp. <i>turumiquitense</i>	(Steyerm.) N. Robson	VE (Sucre)
Brathys	<i>H. cardonae</i>	Cuatrec.	CR (Cartago, San José) VE (Trujillo, Táchira, Mérida, Apuré)
Brathys	<i>H. carinosum</i>	R. Keller	CO (Norte de Santander, Santander) VE (Mérida)
Brathys	<i>H. cassiopifforme</i>	N. Robson	PE (Amazonas)
Brathys	<i>H. chamaemyrtus</i> ssp. <i>chamaemyrtus</i>	Triana & Planchon	CO (Boyacá, Cundinamarca)
Brathys	<i>H. chamaemyrtus</i> ssp. <i>pseudocaracasana</i>	(Steyerm.) N. Robson	VE (Lara, Trujillo)
Brathys	<i>H. costaricense</i>	N. Robson	CR (San José, Cartago, Limon, Puntas Arenas) PA (Chiriquí, CO (Bolívar, Antioquia)
Brathys	<i>H. cuatrecasii</i>	Gleason	CO (Boyacá, Santander)
Brathys	<i>H. cymobrachys</i>	N. Robson	CO (Sierra Nevada del Cocuy)
Brathys	<i>H. decandrum</i>	Turcz.	EC (Cachii to Loja) PE (Amazonas)
Brathys	<i>H. garciae</i>	Pierce	CO (Norte de Santander to Meta) VE (Táchira)
Brathys	<i>H. gleasonii</i>	N. Robson	CO (Norte de Santander, Santander)
Brathys	<i>H. gnidioides</i>	Seemann	CR (San José) PA (Chiriquí)
Brathys	<i>H. goyanesi</i>	Cuatrec.	CO (Cundinamarca, Meta)
Brathys	<i>H. harlingii</i>	N. Robson	EC (Loja)
Brathys	<i>H. horizontale</i>	N. Robson	CO (Santander, Boyacá, Cundinamarca, Meta)
Brathys	<i>H. humboldtianum</i>	Steudel	CO (Valle de Cauca to Norte de Santander) VE (Mérida)

Section	Species ^a	Species authority	Distribution ^b
Brathys	<i>H. traziense</i>	Kuntze ex N. Robson	CR (San José, Cartago, Limón) PA (Chiriquí)
Brathys	<i>H. jaramilloi</i>	N. Robson	CR (Limón) CO (Magdalena, Norte de Santander/César)
Brathys	<i>H. juniperinum</i>	Kunth	VE (Trujillo, Táchira, Mérida) CO (Magdalena/Norte de Santander to Putumayo)
Brathys	<i>H. lancifolium</i>	Gleason	CO (Norte de Santander, Santander, Boyacá) VE (Táchira, Apuré)
Brathys	<i>H. lancioides</i> ssp. <i>congestiflorum</i>	(Triana & Planchon) N. Robson	VE (Mérida) CO (Santander to Cauca)
Brathys	<i>H. lancioides</i> ssp. <i>lancioides</i>	Cuatrec.	CO (Cundinamarca, Meta) EC (Carchi to Cotopaxi)
Brathys	<i>H. laricifolium</i>	Juss.	Western VE along Andes of CO and EC to central PE
Brathys	<i>H. llanganaticum</i>	N. Robson	EC (Tungurahua, Napo)
Brathys	<i>H. loxense</i> ssp. <i>aequatoriale</i>	(R. Keller) N. Robson	EC (Cotopaxi, Chimorazo, Cañar, Azuay, Loja)
Brathys	<i>H. loxense</i> ssp. <i>loxense</i>	Benth.	EC (Chimoborozo, Cañar, southern Azuay, Loja) PE (Cajamarca, La Libertad)
Brathys	<i>H. lycopodioides</i>	Triana & Planchon	CO (Arauca, Boyacá, Cundinamarca)
Brathys	<i>H. magdalenicum</i>	N. Robson	CO (Magdalena) VE (Zulia, Meridá)
Brathys	<i>H. magniflorum</i>	Cuatrec.	CO (Boyacá, Norte de Santander, Santander) VE (Táchira)
Brathys	<i>H. marahuacatum</i> ssp. <i>strictissimum</i>	N. Robson	CO (Boyacá) VE (Mérida)
Brathys	<i>H. martense</i>	N. Robson	CO (Sierra Nevada de Santa Marta)
Brathys	<i>H. matangense</i>	N. Robson	EC (Morona-Santiago)
Brathys	<i>H. mexicanum</i>	L.	VE (Táchira, Mérida) CO (Norte de Santander to Valle de Cauca)
Brathys	<i>H. myricarifolium</i>	Hieron.	CO (Cundinamarca, páramos near Bogotá)
Brathys	<i>H. papillosum</i>	N. Robson	CO (Sierra Nevada del Cocuy)
Brathys	<i>H. parallelum</i>	N. Robson	CO (Norte de Santander/César)
Brathys	<i>H. paramitanum</i>	N. Robson	VE (Trujillo)
Brathys	<i>H. phellos</i> ssp. <i>oroqueanum</i>	N. Robson	CO (Norte de Santander, César)
Brathys	<i>H. phellos</i> ssp. <i>phellos</i>	Gleason	CO (Norte de Santander, Santander) VE (Táchira)
Brathys	<i>H. phellos</i> ssp. <i>platyphyllum</i>	(Gleason) N. Robson	CO (Norte de Santander, Santander)
Brathys	<i>H. pimeleoides</i>	Planchon & Linden ex Triana & Planchon	CO (Norte de Santander/César to Boyacá)

Section	Species ^a	Species authority	Distribution ^b
Brathys	<i>H. prietoi</i>	N. Robson	EC (Azuay)
Brathys	<i>H. prostratum</i>	N. Robson	CO (Cundinamarca, Meta, Boyacá, Santander)
Brathys	<i>H. quitense</i>	R. Keller	EC (Bolívar, Tungurahua, Cañar, Azuay, Loja) PE (Cajamarca, La Libertad)
Brathys	<i>H. recurvum</i>	N. Robson	PE (Chachapoyas, Pasco, Cajamarca)
Brathys	<i>H. ruscoides</i>	Cuatrec.	CO (from Boyacá and Cundinamarca to Putumayo)
Brathys	<i>H. sabiniforme</i>	Trevir.	CO (Boyacá)
Brathys	<i>H. selaginella</i>	N. Robson	CO (Boyacá, Arauca, Cundinamarca)
Brathys	<i>H. simonsii</i>	N. Robson	CO (Sierra Nevada de Santa Marta)
Brathys	<i>H. sprucei</i>	N. Robson	EC (Carchi to Loja) PE (Piura)
Brathys	<i>H. stenopetalum</i>	Turcz.	CO (Santander) VE (Táchira, Mérida)
Brathys	<i>H. strictum</i> ssp. <i>compactum</i>	(Triana & Planchon) N. Robson	CO (Santander, Boyacá)
Brathys	<i>H. strictum</i> ssp. <i>strictum</i>	Kunth	CO (Boyacá, Cundinamarca, Meta)
Brathys	<i>H. struthiolifolium</i>	Juss.	PE (Amazonas, Pasco, Junin, Huancavelica, Cuzco) EC (Zamora-Chinchipe)
Brathys	<i>H. stuebelii</i>	Hieron.	PE (Amazonas, San Martín)
Brathys	<i>H. tetrastichum</i>	Cuatrec.	CO (Boyacá, Santander, VE (Mérida)
Brathys	<i>H. thuyoides</i>	Kunth	CO (Boyacá, Cundinamarca, Meta)
Brathys	<i>H. valleanum</i>	N. Robson	CO (Valle de Cauca)
Trigynobrathys	<i>H. brevistylum</i>	Choisy	EC (Azuay, Imbabura, Pichincha, Bolívar) PE (Ancash, Huánuco, Ayacucho, Apurímac, Cuzco, Piñón) BO (La Paz)
Trigynobrathys	<i>H. killipii</i>	N. Robson	CO (Norte de Santander)
Trigynobrathys	<i>H. relictum</i>	N. Robson	CO (Santander)
Trigynobrathys	<i>H. silenoides</i>	Juss.	PE (Ancash, La Libertad, Cajamarca, Ayacucho, Piñón) EC (all) CO (Antioquia) BO (La Paz)
Trigynobrathys	<i>H. thesitifolium</i>	Kunth	CR (San José) PA (Bocas de Toro) CO (Magdalena) VE (Mérida)

^aTaxonomy according to Robson 1987, Robson 2003. Species in bold are of particular conservation concern

^bCR = Costa Rica, PA = Panama, CO = Colombia, VE = Venezuela, BO = Bolivia, PE = Peru, EC = Ecuador