UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Aspectos químicos e biológicos de espécies de *Hypericum* (Hypericaceae) das secções *Brathys* e *Trigynobrathys* nativas dos Páramos Peruanos

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PORTO ALEGRE, 2014

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Orientador: Profa. Dra. Gilsane Lino von Poser

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RESUMO

Brathys e *Trigynobrathys* (88 e 59 representantes, respectivamente) são as duas maiores seções do gênero *Hypericum* que são distribuídos principalmente na América Central e América do Sul. Das mais de 100 espécies sul-americanas de *Hypericum* quase 65 são endêmicas dos Páramos, ecossistemas de alta altitude, caracterizados por uma vegetação composta principalmente de plantas de roseta gigantes, arbustos e gramíneas, nos quais *Hypericum* é um componente importante. Tendo em vista o escasso conhecimento da fitoquímica destas espécies de *Hypericum*, o presente estudo teve como objetivo estudar a composição fitoquímica e algumas bioatividades de seis espécies de *Hypericum* nativas do Peru (*H. aciculare*, *H. andinum*, *H. brevistylum*, *H. decandrum*, *H. laricifolium* e *H. silenoides*). O material vegetal, seco ao ar, das seis espécies (partes aéreas, caules, folhas e flores), e material vegetal subterrâneo de *H. andinum* (raízes e caules), foram moídas e extraídas por maceração à temperatura ambiente com *n*-hexano. Além disso, foram obtidos extrato etanólicos a partir de quatro espécies (*H. andinum*, *H. brevistylum*, *H. laricifolium* e *H. silenoides*). Os extratos *n*-hexano foram fracionados e as frações foram sometidas a processos cromatográficos obtendo-se cinco derivados de floroglucinol diméricos conhecidos, uliginosina A, uliginosina B, isouliginosina B, hiperbrasilol B e isohiperbrasilol B. Além disso, foram identificadas duas estruturas monoméricas e duas diméricas inéditas em *H. andinum* (raízes) e em *H. laricifolium*, andinina A, hiperlaricifolina A, laricifolina A e laricifolina B. Andinina A mostrou potencial atividade antidepressiva no teste de natação forçada. Do mesmo modo, a atividade antidepressiva dos extratos etanólicos foi avaliada. Estes quatro extratos apresentaram potencial atividade antidepressiva. As análises fitoquímicas por TLC, HPLC-DAD e UPLC-DAD/Q-TOF-MS revelaram que estes extratos são ricos em flavonoides, principalmente hiperosídeo. Os extratos *n*-hexano foram também analisados por um novo método de HPLC-DAD associado a LC-MS e UPLC-Q-TOF-MS . A presença de homólogos superiores M + 14 e regioisómeros foi determinada. A ocorrência natural destes cinco floroglucinois homólogos superiores M + 14 é descrita e a presença de outros compostos identificados pelo padrão de fragmentação MS é apresentada. Estes extratos e o seu principal componente foram capazes de inibir potencialmente a quimiotaxia induzida por LPS. Estes resultados sugerem que os extratos de espécies de *Hypericum* das seções *Brathys* e *Trigynobrathys* são fontes potenciais de novos anti-inflamatórios e antidepressivos.

Palavras-chave: *Hypericum*, Páramos Peruanos, atividade de tipo antidepressiva, atividade antiquimiotática, acilfloroglucinois diméricos, flavonoides.

ABSTRACT

Brathys and *Trigynobrathys* (88 and 59 representatives, respectively) are the two largest sections of the genus *Hypericum* that are principally distributed in Central and South America. Of the more than 100 South American species of *Hypericum* almost 65 are endemic to the Páramos, high-altitude grassland ecosystems characterized by vegetation composed mainly of giant rosette plants, shrubs and grasses, in which *Hypericum* is a prominent component. In view of the scare knowledge on the phytochemistry of these *Hypericum* species, the present research aimed to study the phytochemical composition and some bioactivities of six Peruvian *Hypericum* species (*H. aciculare*, *H. andinum*, *H. brevistylum*, *H. decandrum*, *H. laricifolium* and *H. silenoides*). The air-dried aerial plant material of those six species (stems, leaves and flowers), and underground plant material of *H. andinum* (roots and stems), were ground and extracted by maceration at room temperature with *n*-hexane. Additionally crude ethanolic extracts were obtained from four species (*H. andinum*, *H. brevistylum*, *H. laricifolium* and *H. silenoides*). The *n*-hexane extracts were fractionated, and fractions were further processed by chromatographic procedures to yield five known dimeric acylphloroglucinol derivatives uliginosin A, uliginosin B, isouliginosin B, hyperbrasilol B and isohyperbrasilol B. In addition, two monomeric and two dimeric acylphloroglucinol structures were identified in *H. andinum* (roots extract) and *H. laricifolium* for the first time, andinin A, hyperlaricifolin A, laricifolin A and laricifolin B. Andinin A showed potential antidepressant-like activity in the forced swimming test. Similarly, the antidepressant-like activity of the crude ethanolic extracts was assessed. These four extracts possessed a potential antidepressant-like activity. The phytochemical analyses by TLC, HPLC-DAD and UPLC-DAD/Q-TOF-MS revealed that the extracts were rich in flavonoids, principally hyperoside. The *n*-hexane extracts were also analyzed by a new HPLC-DAD fingerprint method associated with LC-MS and UPLC-Q-TOF-MS. The presence of M + 14 higher homologues and regioisomers could be distinguished. The natural occurrence of these five M + 14 higher homologues is described and the presence of other compounds identified by their MS fragmentation pattern is presented. These extracts and their main dimeric acylphloroglucinol component were able to potently inhibit the LPS-induced chemotaxis on rat PMN. These results suggest that extracts of *Hypericum* species from sections *Brathys* and *Trigynobrathys* are potential sources of new anti-inflammatory and antidepressant molecules.

Keywords: *Hypericum*, Peruvian Páramos, antidepressant-like activity, antichemotactic activity, dimeric acylphloroglucinols, flavonoids.

SUMÁRIO

INTRODUÇÃO GERAL

As espécies do gênero *Hypericum* (Hypericaceae) têm uma distribuição principal em regiões temperadas, mas também podem ser encontradas em regiões tropicais do mundo, particularmente na América do Sul e na África (ROBSON, 2003). A Cordilheira Americana é uma série quase ininterrupta de uma gama de montanhas que se encontra do Alasca aos Andes, que se estende para o extremo sul da América do Sul. Os picos mais elevados encontrados na Costa Rica e Panamá pertencem à Cordilheira de Talamanca, já os picos mais elevados na Colômbia, Equador, Venezuela, Peru e Bolívia, pertencem à Cordilheira dos Andes da América do Sul (CROCKETT *et al*., 2010). Nesses países, na faixa de altitudes entre 3000 - 5000 m, acima da linha das árvores, mas abaixo da linha de neve, encontra-se um tipo de vegetação específico chamado de Páramo, caracterizado por grandes Asteraceae (tais como *Espeletia*), almofadas (*Werneria*), gramíneas (*Calamagrostis*) e arbustos verdes espalhados (incluindo *Hypericum*). O conhecimento sobre as espécies de *Hypericum* que ocorrem nessas regiões ainda é disperso e escasso, em parte devido à elevada proporção de endemismo e os resultantes padrões de distribuição restritos (CROCKETT *et al*., 2010).

As quase 500 espécies de *Hypericum* de ocorrência no mundo inteiro foram divididas em 36 seções taxonômicas com base em características morfológicas (ROBSON, 2003; ROBSON, 2012). A maioria das espécies de *Hypericum* nativas das regiões montanhosas na América do Sul pertence à seção taxonômica *Brathys* e, em menor extensão, *Trigynobrathys*. A seção *Brathys* compreende 88 espécies de arbustos (raramente árvores pequenas) subarbustos e ervas anuais pequenas (raramente perenes) distribuídas principalmente em dois centros: Belize e Cuba, e a fronteira da Venezuela com a Colômbia. A partir desta ultima área tem se irradiado espécies, principalmente arbustivas, para o leste de Roraima (Brasil), para o oeste de Costa Rica, e ao sudoeste ao longo dos Andes e Bolívia. Igualmente, a seção *Trigynobrathys* inclui 59 espécies de arbustos, subarbustos, bem como ervas perenes e anuais.

A principal área de especiação é o sudeste do Brasil, com uma radiação primária para o norte e leste do Brasil, sudoeste dos EUA, sul do Uruguai e norte da Argentina, ao oeste da Bolívia e do Peru, e norte do Chile e daí para o norte ao longo da Cordilheira dos Andes, ao sul da Colômbia e as Ilhas Galápagos, para a Nova Zelândia, Austrália, Nova Caledônia e Nova Guiné e ocorrência dispersa no sudoeste da Ásia (ROBSON; 1987; ROBSON; 1990, ROBSON, 2012).

Nas regiões montanhosas dos Páramos Peruanos têm sido relatadas pelo menos 14 espécies de *Hypericum* (CROCKETT *et al*., 2010), sendo que as espécies *H. aciculare* Kunth (**Figura 1**), *H. andinum* Gleason (**Figura 2**), *H. decandrum* Turcz (**Figura 3**), *H. laricifolium* Juss , (**Figura 4**) (seção *Brathys*), *H. brevistylum* Choisy (**Figura 5**) e *H. silenoides* Juss (**Figura 6**), (seção *Trigynobrathys*) têm sido relatadas em diversas áreas geográficas do Peru.

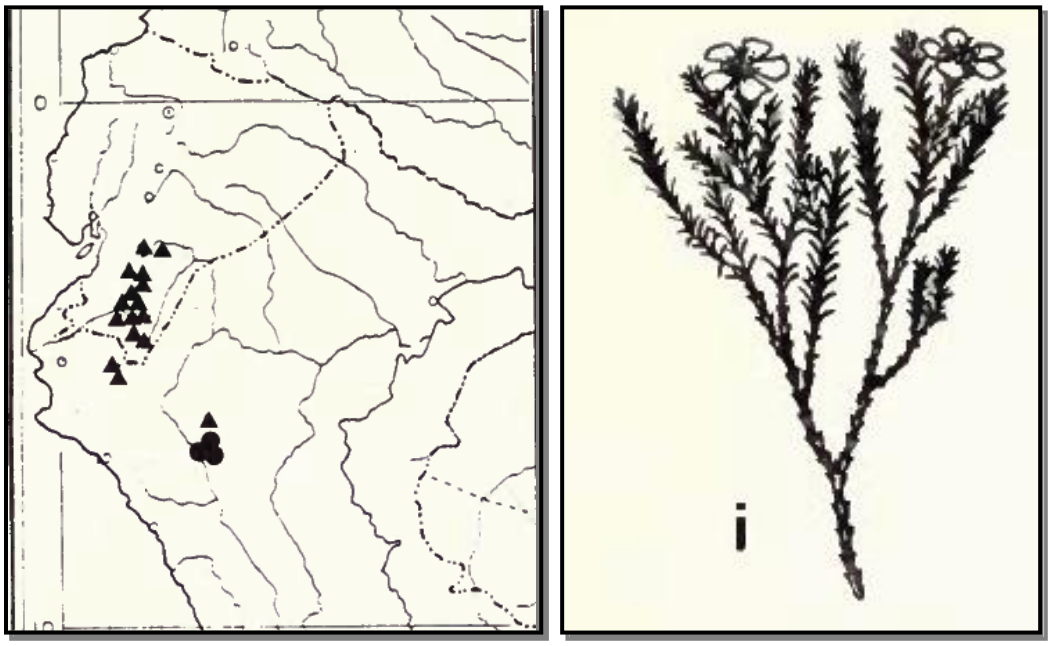

Figura 1. Distribuição e representação de *Hypericum aciculare* (▲): Equador (Azuay, Loja), Peru (Piura, Amazonas) (ROBSON, 1987). **Nomes comuns e usos tradicionais**: No distrito de Huancabamba a planta é conhecida como 'Hierba de las Cordilleras', 'Lechuguilla', 'Hierba de Iman'. As folhas e flores são utilizadas em febre e processos inflamatórios (BUSSMANN e GLENN, 2010).

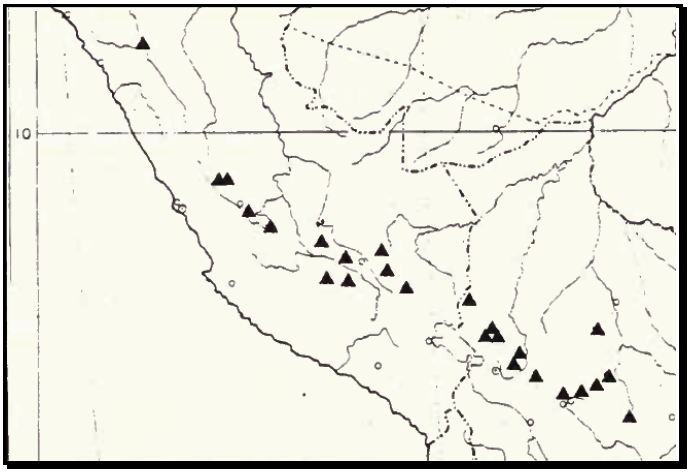

Figura 2. Distribuição de *Hypericum andinum* (▲): Peru (La Libertad, Junín, Huancavelica, Cusco, Puno), Bolívia (La Paz, Cochabamba, Santa Cruz) (ROBSON, 1987). **Nomes comuns e usos tradicionais**: Não há relatos na literatura. Na localidade de Amparaes é conhecida como 'Chinchimali', as raízes bem como as folhas e flores são utilizadas em cozimento como anti-inflamatório renal (comunicação pessoal com habitantes locais).

Figura 3. Distribuição e representação de *Hypericum decandrum* (●): Equador (Em localidades dispersas de Carchi a Loja), Peru (Amazonas) (ROBSON, 1987). **Nomes comuns e usos tradicionais**: Não há relatos na literatura.

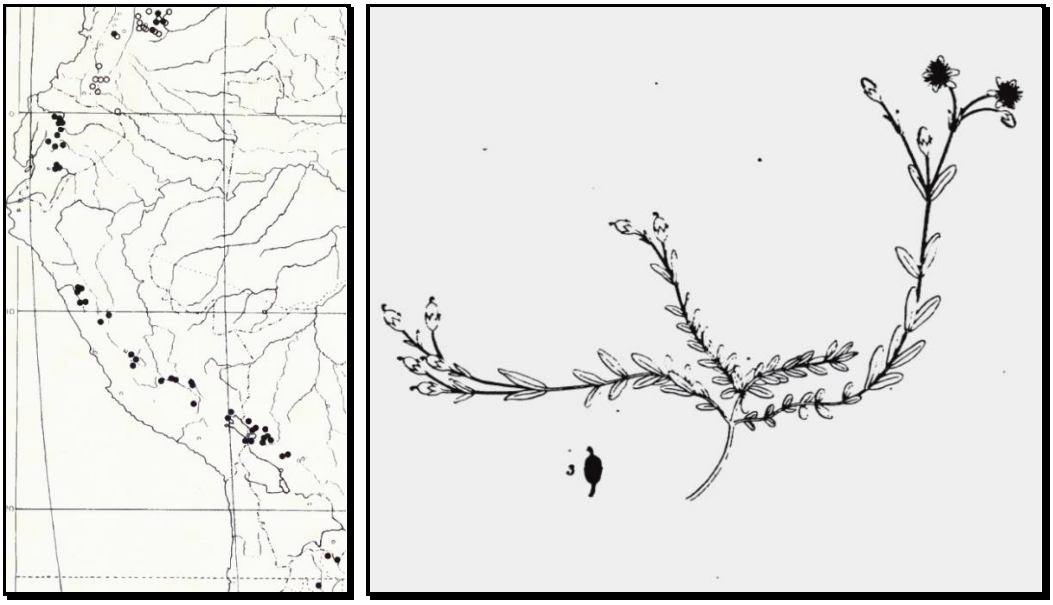

Figura 4. Distribuição e representação de *Hypericum brevistylum* (●): Colômbia (Cundinamarca, Valle de Cauca), Equador, Peru (Huánuco e Ancash até Puno), Bolívia (La Paz, Cochabamba, Tarija), Argentina (Jujuy e Salta) (ROBSON, 1990). **Nomes comuns e usos tradicionais**: Não há relatos na literatura.

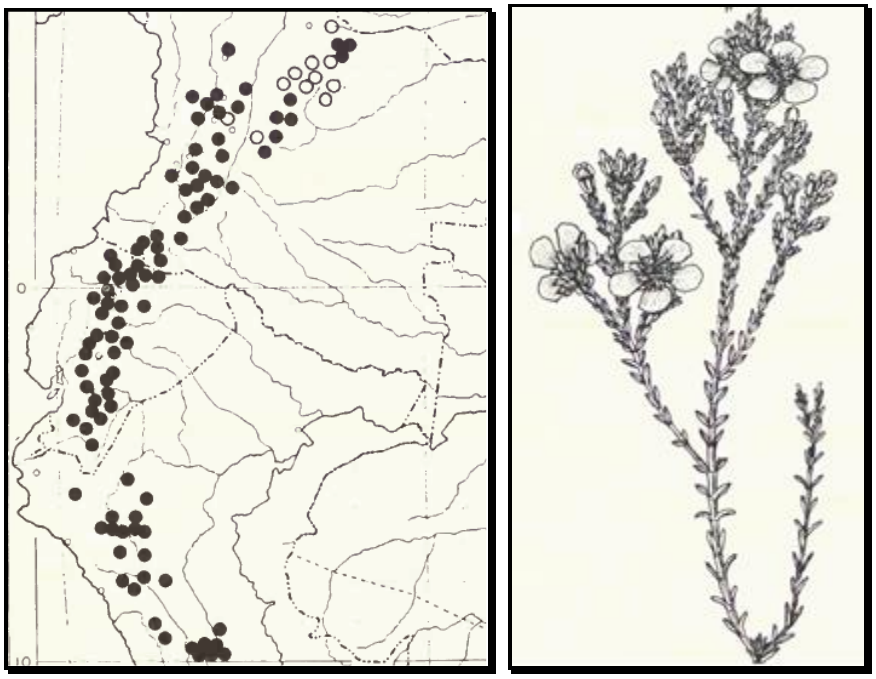

Figura 5. Distribuição e representação de *Hypericum laricifolium* (●): Desde o oeste da Venezuela (Lara, Trujillo, Mérida, Zulia), através da cordilheira central e oriental da Colômbia e Equador até o Peru (Huánuco, Ancash) (ROBSON, 1987). **Nomes comuns e usos tradicionais**: No departamento de Cajamarca esta espécie é conhecida como 'Chinchanga ou Chinchango' (BUSSMANN *et al*., 2010). A decocção de talos e folhas é utilizada para tingir de cor amarela o algodão e a lã; também é utilizada para o tratamento de verrugas tópicas.

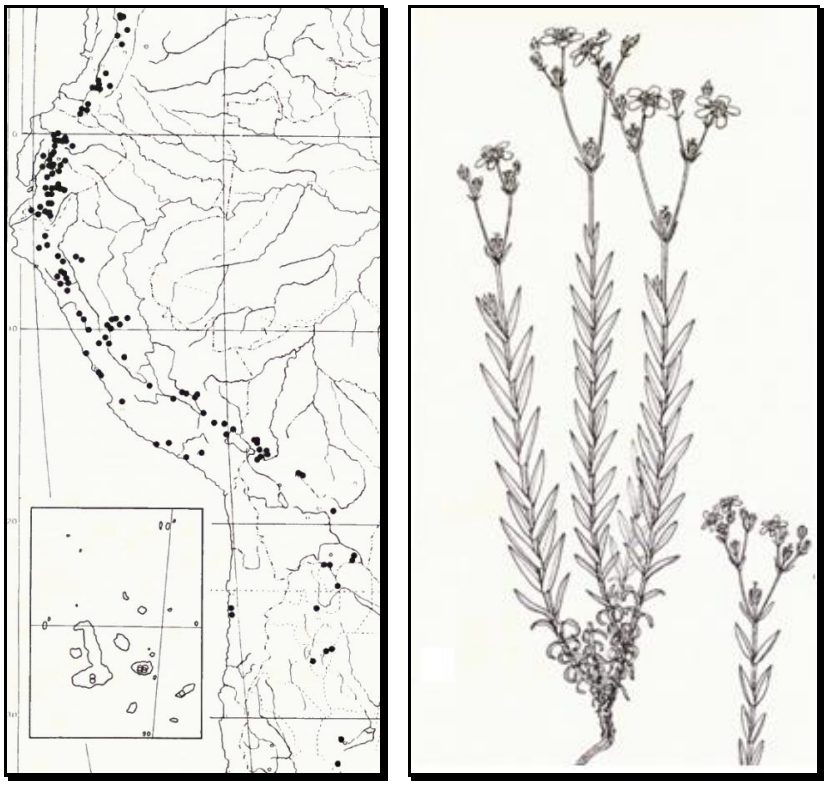

Figura 6. Distribuição e representação de *Hypericum silenoides* (●): Chile (Antofagasta), Peru (Arequipa até Lima, Cajamarca e Piura até Cuzco), Equador, Colômbia (Nariño, Cauca, Antioquia), Bolívia, Argentina (Jujuy até Córdoba) (ROBSON, 1990). **Nomes comuns e usos tradicionais.** No distrito de Morropón, departamento de Piura é conhecida como "corazoncillo" e "hierva de la rabia". No vale do rio Ocoña, distrito de Parinacochas, departamento de Ayacucho é conhecido como "sunchito". Seus usos não tem sido relatados.

Em contraste com o amplamente utilizado *H. perforatum*, as espécies das regiões dos Páramos da América Central e América do Sul têm sido raramente examinadas do ponto de vista químico (CROCKETT *et al*., 2010). No entanto, vários estudos de espécies, pertencentes às seções *Brathys* e *Trigynobrathys* que ocorrem em altitudes mais baixas no Sul do Brasil foram publicados. Estes incluem relatos da presença de flavonoides, xantonas e derivados de floroglucinol a partir das folhas e flores de *H. brasiliense* Choisy (ROCHA *et al*., 1994, ROCHA *et al*., 1994; ROCHA *et al*., 1996), benzopiranos das partes aéreas de *H. polyanthemum* Klotzsch ex Reichardt (FERRAZ *et al*., 2001), derivados de floroglucinol de *H. myrianthum* Cham. & Schlecht. (FERRAZ *et al*., 2002a), *H. carinatum* Griseb., *H. polyanthemum, H. caprifoliatum* Cham. & Schlecht. e *H. connatum* Lam. (NÖR *et al*., 2004), derivados de benzofenona de *H. carinatum* (BERNARDI *et al*., 2005) e flavonoides de *H.*

ternum A. St.-Hil (BERNARDI *et al*., 2007). Além disso, taninos e óleos essenciais foram pesquisados em algumas espécies (DALL'AGNOL *et al*., 2003; FERRAZ *et al*., 2005a) e a ausência de hipericinas foi verificada em oito espécies analisadas pelos métodos cromatográficos de TLC e HPLC (FERRAZ *et al*., 2002b).

Em virtude da escassez de dados na literatura sobre a composição química das espécies do gênero *Hypericum* dos Páramos, o presente estudo teve como objetivo geral o estudo de seis espécies de *Hypericum* nativas do Peru, com ênfase no isolamento e identificação de compostos fenólicos assim como a determinação de algumas atividades biológicas. Visando um ordenamento dos assuntos abordados, o trabalho está dividido em quatro capítulos:

O Capítulo 1 apresenta uma revisão da ocorrência e as propriedades analíticas mais importantes de derivados acilfloroglucinol diméricos, compostos fenólicos relatados para o gênero *Hypericum*, apenas nas seções *Brathys* e *Trigynobrathys*. Além disso, apresenta-se resultados de isolamento de derivados acilfloroglucinol diméricos conhecidos, mas obtidos de novas fontes, em extratos lipofílicos de quatro espécies de *Hypericum* nativas dos Páramos do Peru. O significado quimiotaxonômico destes achados é discutido.

O Capitulo 2 apresenta o isolamento e elucidação estrutural por técnicas espectroscópicas (UV-Vis, 1D e 2D NMR) e espectrométricas (MS) de derivados acilfloroglucinol inéditos a partir de extratos lipofílicos das espécies *H. andinum* e *H. laricifolium*. Além disso, são apresentados os resultados da avaliação de um dos compostos inéditos isolados no ensaio de atividade antidepressiva (FST).

O Capítulo 3 apresenta resultados de ensaios de atividade antidepressiva de extratos brutos etanólico de quatro espécies de *Hypericum* nativas do Peru por meio do teste de natação forçada em camundongos (FST). Adicionalmente, apresenta-se a caracterização qualitativa e quantitativa de flavonoides e derivados acylphloroglucinol diméricos nos extratos por meio de HPLC e UPLC. Além disso, a composição

fitoquímica de espécies de *Hypericum* com atividade antidepressiva é brevemente resumida e ressalta-se a importância dos flavonoides para esta atividade.

O Capitulo 4 apresenta resultados de ensaios de atividade antiquimiotática de extratos lipofílicos de seis espécies de *Hypericum* nativas do Peru. Adicionalmente, apresenta-se a caracterização qualitativa e quantitativa de derivados acilfloroglucinol diméricos nos extratos por médio de HPLC e UPLC, com o auxilio de análise de componentes principais (PCA) e construção de cladogramas (HCA). Além disso, apresenta-se a caracterização de homólogos M+14 inéditos de compostos já conhecidos, uliginosina A, uliginosina B, isouliginosina B, hiperbrasilol B e isohiperbrasilol B por médio de técnicas cromatográficas (HPLC, UPLC), espectroscópicas (UV-Vis) e espectrométricas (MS).

CAPÍTULO I

"Dimeric acylphloroglucinols in *Hypericum* species from sections *Brathys* and *Trigynobrathys*"

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Dimeric acylphloroglucinols in *Hypericum* species from sections *Brathys* and *Trigynobrathys*

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Abstract

Hypericum is a prolific source of acylphloroglucinol derivatives. Unlike the monomeric polyisoprenylated acylphloroglucinols hyperforin and adhyperforin, which are the main phloroglucinols in *Hypericum perforatum* (section *Hypericum*), dimeric structures are to the best of our knowledge exclusively reported in sections *Brathys* and *Trigynobrathys* belonging to the genus *Hypericum*. Their occurrence, as well as the analytical properties of the thirty-one dimeric acylphloroglucinols currently reported for *Hypericum* spp. are reviewed. Additionally, the presence of dimeric acylphloroglucinol in four Peruvian *Hypericum* species is presented and their chemotaxonomic significance explored.

Keywords

Chemotaxonomy; isolation; NMR spectroscopy; tautomerism; translucent glands

Introduction

Hypericum L. (Hypericaceae) is a genus of nearly 500 species of small trees, shrubs and herbs currently distributed in 36 taxonomic sections. The species of this genus occur in all

temperate parts of the world with main centers of diversity in Eurasia and Andean South America (Robson 2012). They are absent from habitats that are extremely dry, hot or cold, and in the tropics are almost always confined to high elevations (Robson 2003).

Hypericum perforatum L. (St. John's wort) is one of the most important medicinal herbs of the genus. Their extracts are taken for their reported activity against mild to moderate depression (Kasper et al. 2010). In addition to *H. perforatum* many other species of the genus have been incorporated in traditional medicine systems in many countries around the world (Guedes et al. 2012), are sold as ornamentals (Crockett and Robson 2011), or may play other economically important rules as source of firewood and dye plants(Crockett et al. 2010).

Brathys and *Trigynobrathys* are the two largest sections of the genus *Hypericum* with 88 and 59 representatives, respectively. They are principally distributed in South America, from southeast Brazil, Uruguay, north Argentina and central Chile northward through the Andes from Bolivia, Peru and Venezuela to Central America and on the Galapagos Islands and Cuba, to a lesser extent representatives are found in North America, tropical and eastern Asia (central China, across the Himalayas to southern India and Sri Lanka), and in the Pacific Region from north Japan to New Guinea, New Caledonia, Australia and New Zealand (Robson 2012).

Chemical investigations of members of these two sections have revealed that these species are prolific sources of flavonoids, xanthones, dimeric acylphloroglucinols and less frequently monomeric acylphloroglucinols, benzopyrans and benzophenones (Crockett and Robson 2011; Crockett 2012; Barros et al. 2013). Many of these metabolites have valuable biological activities as antibacterial, antioxidant, cytotoxic, and inhibition of monoamine oxidase (Gnerre et al. 2001; Bernardi et al. 2005; Franca et al. 2009; Pinhatti et al. 2013). In an attempt to assess the medicinal properties some species of *Hypericum* from *Brathys* and *Trigynobrathys* sections, as in *H. perforatum*, these species were screened for antidepressant and analgesic activities (Daudt et al. 2000; Gnerre et al. 2001; Mendes et al. 2002). Even though the ethnopharmacological use of these plants as remedies for central nervous system-related illnesses has been rarely reported (Noeli 1998), this approach was helpful in the search for alternative sources of new antidepressant and analgesic molecules. Some dimeric acylphloroglucinols have been revealed to display innovative mechanisms of action (Viana et

al. 2005; Viana et al. 2006; Sakamoto et al. 2012) and provide promising molecular scaffolds for the development of new antidepressant and analgesic drugs (Stein et al. 2012; Stolz et al. 2012).

Unlike the polyisoprenylated monomeric phloroglucinols hyperforin and adhyperforin from *H. perforatum*, species of *Hypericum* from the sections *Brathys* and *Trigynobrathys* are sources of primarily dimeric structures consisting often of a filicinic acid and a phloroglucinol moiety linked by a methylene bridge. They possess acetyl, *n*-propionyl, *iso*-butyryl or 2-methylbutyryl functionalities attached to the two carbocyclic rings. Usually a benzopyran ring skeleton forms a part of the phloroglucinol moiety, and a C-prenyl side chain may be either includes in the filicinic acid or phloroglucinol moiety, in both of them or also attached to the benzopyran ring. The presence of O-prenyl, C-geranyl and benzoyl functionalities attached to the phloroglucinol moiety is more uncommon. Until now, the isolation of 28 unique structures has been reported in the literature (Barros et al. 2013) and an other three have been recently identified using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) and quadrupole orthogonal time-of-flight (Q-TOF) mass spectrometry (Crispin et al. 2013) (**Fig. 1**) in 19 species of *Hypericum* from sections *Brathys* and *Trigynobrathys* investigated for dimeric acylphloroglucinols (Barros et al. 2013; Crispin et al. 2013). Even though this data reveals a high degree of chemical diversity, the low yield, instability and difficulties in the process of isolation and identification of these compounds (Glisic et al. 2008; Barros et al. 2011; Crispin et al. 2013) are some factors that have perhaps justified their limited exploration.

In view of the high value and promising activities of dimeric acylphloroglucinols as antidepressant, analgesic and cytotoxic drug leads (Stein et al. 2012; Stolz et al. 2012; Pinhatti et al. 2013), the identification of new sources and novel structures of compounds of this class is of high interest, not only in *Hypericum* but also in other phloroglucinol-producing genera, e.g. *Elaphoglossum* (Socolsky et al. 2012a). The main chemical and analytical properties of dimeric acylphloroglucinols in *Hypericum* species from sections *Brathys* and *Trigynobrathys* with an especial emphasis on their structure elucidation using nuclear magnetic resonance (NMR) spectroscopy, are reviewed. Additionally, the isolation of some dimeric acylphloroglucinols from *H. andinum*, *H. brevistylum*, *H. laricifolium* and *H. silenoides*, species from the Andean Páramos, is presented.

Translucent glands and dimeric acylphloroglucinol distribution

The presence of translucent glands is common to all *Hypericum* species, while dark glands (nodules) are found in about 2/3 species of the genus (Crockett and Robson 2011). Phloroglucinols and naphthodianthrones, respectively, are biosynthesized in secretory cells delimiting these two types of glands. (Zobayed et al. 2006; Soelberg et al. 2007; Crockett and Robson 2011). Even though there is a positive correlation between the size and number of dark glands and the overall content of the naphthodianthrone hypericin in *H. perforatum* (Zobayed et al. 2006), this correlation could not be made for hyperforin and the translucent glands. Nonetheless, morphology, phenology and population metabolite variation studies carried out in *H. perforatum*, *H. montbretii, H. orientale,* as well as other related species, indicate that the content of this metabolite is highly variable. Concentrations of hyperforin have been seen to increase during the course of ontogenesis, with the highest levels during the floral and fresh fruiting developmental stages. Additionally, open flowers and ripe fruits are usually described to have higher amounts as compared to stems, leaves and other reproductive parts (Cirak and Radusiene 2007; Cirak et al. 2008; Cirak et al. 2012; Tekel'ová et al. 2000).

In *Hypericum* species from section *Brathys* and *Trigynobrathys,* only translucent glands are found (Robson 1987; Robson 1990). The nature, ontogeny and distribution pattern of translucent glands in *H. gentianoides* (*Brathys* section) have been surveyed, showing that these glands develop schizogenously and undergo major morphological and biochemical changes over time and space. Additionally, the glandular content was isolated by a capillary needle method and analyzed by ESI-MS, showing that these glands contained primarily dimeric acylphloroglucinols **12**, **13** and **19** (Babka 2009). These findings support the hypothesis that the glands are the primary site for the storage of dimeric acylphloroglucinols. Furthermore, it was shown that these compounds were distributed in most above-ground organs of the flowering plant, with a preferential accumulation in the flower buds and young leaves of *H. gentianoides* (Babka 2009; Babka et al. 2010).

However, dimeric acylphloroglucinols have also been isolated from roots, where no translucent glands are present. In the case of *H. drummondii*, compounds **1**, **2**, **4**, **5** and **6** were isolated from roots, while **7**, **10** and **11** from aerial parts (Jayasuriya et al. 1989; Jayasuriya et

al. 1991). Similar variations in acylphloroglucinols were observed in *H. ternum* in which **14** was readily isolated in roots, while the aerial parts afforded only terpene-like compounds; **3** and **14** were isolated from both roots and aerial parts of *H. myrianthum* (Bernardi 2007). This variation may also be influenced by variability among population and phenology as in the case of an accession of *H. brasiliense* that showed a high content of **16** in the roots, especially at the flowering stage, but not in the shoots at all developmental stages (Abreu et al. 2004), even though **16** had been previously isolated from the leaves of plants from a different population of *H. brasiliense* (Rocha et al. 1995). These variations might be explained by several factors, from the endogenous regulation of physiological processes to environmental characteristics. Taking in consideration these aspects could be helpful in the selection of materials during future study of the phytochemistry of *Hypericum* species from sections *Brathys* and *Trigynobrathys*.

Isolation and identification of dimeric acylphloroglucinols in *Hypericum* spp.

Extraction and isolation

Dimeric acylphloroglucinols are lipophilic compounds usually extracted from aerial plant parts material by percolation or maceration with petrol, ether, *n*-hexane or methanol (references in **Table 1**). Despite the position of four to five polar hydroxyl groups (**Fig. 1**), the compounds are quite lipophilic which may be explained, at least in part, by the presence of neighbouring carbonyl and hydroxyl groups, allowing the formation of intramolecular hydrogen bonds (Äyräs et al. 1981; Mammino and Kabanda 2009). When extracted with ether or methanol, this extract can be further fractioned with *n*-hexane yielding a dark or greenish viscous oil residue. This residue may be further treated with acetone to obtain an insoluble fatty residue, containing undesirable epicuticular waxes, and an acetone-soluble fraction rich in dimeric acylphloroglucinols that is further subjected to chromatographic procedures. Moreover, it is noteworthy to mention the use of supercritical fluid extraction with CO_2, considered to be a cleaner and feasible alternative technology to solvent extraction for *Hypericum*. This method has been successfully applied for the selective extraction of benzopyrans, benzophenones and dimeric acylphloroglucinols from *H. polyanthemum* (Cargnin et al. 2010), *H. carinatum* (Barros et al. 2011) and *H. caprifoliatum* (Almeida et al. 2013).

Column chromatography on silica gel, usually prepared with *n*-hexane and developed with chloroform, using mixtures of increasing polarity of chloroform/methanol, *n*-hexane/dichloromethane, *n*-hexane/ethyl acetate or *n*-hexane/ethyl ether with 0.25% formic acid, is usually the first step of crude extract fractionation. Final purification steps often involve the use of countercurrent chromatography, centrifugal planar chromatography, gel filtration over sephadex LH-20, reverse phase medium pressure chromatography and/or repeated column chromatography with silica gel (references in **Table 1**). The use of preparative high performance liquid chromatography (HPLC) for the isolation of dimeric acylphloroglucinols from *Hypericum* species of section *Brathys* and *Trigynobrathys* is not frequently reported, although it was used to fractionate a methanol extract of *H. gentianoides* into flavonoid- and -acylphloroglucinol rich fractions (Hillwig et al. 2008), and to obtain acylphloroglucinol fractions from an ethanolic extract of *H. gentianoides* (Huang et al. 2011).

Crystallization

Dimeric acylphloroglucinols are usually isolated as colorless crystals (e.g. **1**, **2**) or yellow powders (e.g. **19**, **20**, **21**, **24**) from *n*-hexane, as yellow crystals from *n*-hexane (e.g. **4**, **5**, **6**, **9**), methanol (e.g. **15**) or pentane (e.g. **12**), as yellow platelets from nitromethane (e.g. **14**), as yellow needles from methanol (e.g. **13**, **17**, **26**) or acetonitrile (e.g. **15**, **16**), and sometimes recrystallized from mixtures of ethanol-ethyl acetate (e.g. **23**) and acetonitrile-chloroform (4:1) (e.g. **12**) (references in **Table 1**).

Ultraviolet (UV) spectroscopy

Many dimeric acylphloroglucinols have characteristic three-peaked UV spectra with maxima around 220, 300, and 350 nm or 226, 287, and 357 nm (**Table 1**). These characteristic peaks have been used as an indicative of acylphloroglucinols (Hillwig et al. 2008; Crispin et al. 2013).

Infrared (IR) spectroscopy

The IR spectra of almost all dimeric acylphloroglucinols show broad abortion bands in the 3000-3500 cm^{-1} range related to the O-H stretching of intramolecularly chelated hydroxyl protons, and intense peaks between 1600-1650 cm^{-1}, which are signals that arise from the presence of an enolic *β*-triketo system (references in **Table 1**).

Electron impact (EI) and electrospray ionization (ESI) mass spectrometry (MS)

The EI-MS reported data of dimeric acylphloroglucinols (**Table 1**) shows that these compounds have a characteristic fragmentation pattern. The methylene bridge that connects the filicinic acid and the phloroglucinol moiety is the main fragmentation target, resulting in molecule cleavage into two roughly equal pieces. Therefore, in addition to the molecular ion, EI-MS spectra reveal the *m*/*z* of the fragments composing the diacylphloroglucinol, thus potentially revealing the substitution pattern of each moiety. The primary EI-MS fragmentation pattern and major fragments expected for dimeric acylphloroglucinols is exemplified by drummondin C in **Fig. 2**. When a benzopyran ring skeleton is present as part of the phloroglucinol moiety (e.g. **4**, **5**, **6**, **14**, **15** and isomers), characteristic fragment (*m*/*z* 219, *m*/*z* 233, *m*/*z* 247) is stabilized by the loss of a methyl radical from the dimethyl chromene system (Parker an Johnson 1968; Jayasuriya et al. 1989). The fragmentation pattern of dimeric acylphloroglucinols submitted to ESI-MS greatly resembles that seen with EI-MS. Since EIS-MS is a less aggressive technique, the fragments observed with this method come from the molecule cleavage at the methylene bridge, and many characteristic fragments seen with EI-MS are lacking with EIS-MS, e.g. the ion of prenyl side chains (*m*/*z* 69) were not observed in the ESI-MS spectrum of **12** and **13** (Crispin et al. 2013).

Nuclear magnetic resonance (NMR) spectroscopy

The ^{1}H and ^{13}C NMR published data of dimeric acylphloroglucinols are shown in **Table 2-6**. These data were gathered from their original references and organized by reassignment of the carbon skeleton numbering scheme of the molecule structures as shown in **Fig. 1** in order to simply the visualization of common signal features.

^{1}H NMR spectra of dimeric acylphloroglucinols are highly informative, containing several interesting features that reveal structural details. As a first insight, the signals that arise from

protons present at the methylene bridge and/or hydroxyl protons are highly suggestive of this kind of compound. The presence of characteristic methylene protons (δ 3.46-3.60) at the linkage between the filicinic acid and the phloroglucinol moiety is a common feature to almost all dimeric acylphloroglucinols. This signal, appearing as a singlet or sometimes as a broad singlet, may be resolved into two independent singlets (e.g. **1**, **2** and **23**) or a set of two doublets (e.g. **18**) in spectra acquired at a high working frequency.

Another interesting aspect of the ^{1}H NMR spectra of dimeric acylphloroglucinols is the presence of singlets in a very low field (δ 19.40-9.00; exceptionally δ 6.40 in **12**, δ 6.41 in **22** and δ 6.42 in **11**), each accounting for an exchangeable or chelated hydroxyl groups. These signals are absent from the spectrum when the sample has been shaken with deuterium oxide (Parker and Johnson 1968). The peak at ~ δ 18.00 ppm (e.g. δ 18.68 in **14**), at a remarkably low position, has proved to be very informative since enolizable β-triketones are one of the few systems known to resonate in this region. This observation, together with the fact that geminal methyl groups in the filicinic acid moiety display singlet or broad singlet signals at δ 1.45-1.59 ppm (exceptionally δ 1.19-1.28 ppm in **19**, **20**, **21** and **22**), further characterize the acylfilicinic acid moiety.

The presence of protons in simple and branched side chains give rise to signals that are indicative of acetyl (e.g. in **1**, δ 2.70, *s*), *n*-propionyl (e.g. in **2**, δ 3.20, *q*; δ 1.18, *t*), *iso*-butyryl (e.g. in **3**, δ 4.20, *sep*; δ 1.18, δ 1.18, *d*) and 2-methylbutyryl (e.g. in **20**, δ 4.07, *sex*; δ 1.11, *d*; δ 1.38, 1.81, *m*; δ 0.88, *t*) functionalities attached to both the filicinic acid and the phloroglucinol moieties. Variations in the length of the acyl side chains give rise to homologues that are readily distinguishable, demonstrated in **1**, **2**, **3**, also **4**, **5**, **6**, and **19**, **20**, **21**.

C-isoprenylation is revealed by signals corresponding to protons within a methylene, a methine and two geminal methyl groups. C-isoprenyl groups in dimeric acylphloroglucinols are usually attached to the filicinic acid moiety (e.g. in **13**, δ 2.41, *br d*; δ 4.81, *br t*; δ 1.39, δ 1.44, *br s*), the phloroglucinol moiety (e.g. in **12**, δ 3.45, *d*; δ 5.22 *pseudo t*; δ 1.79, δ 1.85 *s*), to both (e.g. in **11**), or sometimes attached to the benzopyran ring moiety (e.g. in **25**, **26** and **27**). O-isoprenyl side chains have been also described in **10** (δ 4.81, *d*; δ 5.64 *br t*; δ 1.86, δ

1.86 *s*) and **23** showing more highly deshielded signals than in C-isoprenyl side chains, especially as exemplified by the signal of methylene involved in the ether linkage.

The presence of a 2,2-dimethyl chromene moiety is revealed by a characteristic pair of doublets that come from olefinic protons (e.g. in **4**, δ 5.44, *d*; δ 6.69, *d*) and the protons of two geminal methyl groups (e.g. in **4**, δ 1.49 *br s*; δ 1.49 *br s*). The attachment of a C-isoprenyl group to one of the geminal methyl groups of the base 2,2-dimethyl chromene moiety results in the formation of an isoprenylmethyl-methyl-chromene system that displays an additional methylene signal (δ 1.75-1.82, *m*) as shown in **25**, **26** and **27**.

Signals displayed by a mono substituted benzene ring (δ 7.35-7.64, *m*) are exemplified by **23** and **24**. Aromatic proton signals from the phloroglucinol moiety (δ 6.02-6.20, *s*) are also observed in **10** and **23**. C-geranyl side chains are revealed as a set of three geminal methyl, three methylene and two methine signals as in **22** and **24** (**Table 4**).

The reliability of the hydroxyl ^{1}H NMR signals for the determination of the cyclisation pattern of the 2,2-dimethyl chromene system has been explored and confirmed by selective insensitive nuclei enhanced by polarization transfer (SINEPT) experiments on **6** (3-OH, δ 9.94; 5-OH, δ 18.42; 5'-OH, δ 11.47; 7'-OH, δ 15.88 *s*) and **8** (3-OH, δ 9.04; 5-OH, δ 18.47; 7'-OH, δ 11.64; 9'-OH, δ 14.06 *s*), two isomeric compounds that differ each other in the orientation of the pyran ring relative to the rest of the molecule. The SINEPT technique resolve this problem by inducing polarization transfer through the slow exchange chelated hydroxyl groups on the chromene moiety of these compounds, enabling the localization of hydroxyl groups in the chromene moiety and, hence, the orientation of the pyran ring (Jayasuriya et al. 1994). Correlation experiments (homonuclear correlation spectroscopy, COSY; heteronuclear single-quantum correlation spectroscopy, HSQC; heteronuclear multiple-bond correlation, HMBC) can provide details that help determine the substitution pattern of the acylphloroglucinol and acylfilicinic acid moieties, as well as the cyclization pattern of dimeric acylphloroglucinols with a benzopyran ring skeletal element. The structures of elaphogayanin A, yunguensin A and yunguensin F have recently been determined (Socolsky et al. 2010a; Socolsky et al. 2010b).

The ^{13}C NMR spectra of dimeric acylphloroglucinols display many useful signals. A characteristic C-7 methylene bridge carbon signal is displayed at δ 16.8-19.1 ppm; whereas the carbonyl carbon signals, C-8 and C-11', of the acetyl (δ 203.0-205.1), *n*-propionyl (δ 206.9-207.1), *iso*-butyryl (δ 209.9-212.3) and 2-methylbutyryl (δ 208.3-208.9) functionalities are displayed in a characteristic low field ranges. A third C-1 carbonyl carbon signal located in the filicinic acid moiety is usually seen between δ 195.5-200.1; although this signal may have been missassigned because early structure elucidation works were poorly supported by correlation spectra, and thus this value corresponds to C-5, as supported by HBMC and observed in many fern dimeric acylphloroglucinols (Socolsky et al. 2012a); e.g. for **14** and **16** in **Fig. 3**.

Furthermore, the filicinic acid moiety displays four characteristic enol carbon signals, C-2 (δ 105.8-111.0), C-3 (δ 170.0-183.9), C-5 (δ 187.2-198.9; similarly this value corresponds to the C-1), and C-6 (δ 105.0-114.4) and a quaternary carbon signal, C-4 (dimethyl substituted δ 44.0-44.8; exceptionally δ 53.6-53.7 as in **19**, **20** and **21**). This latter signal is characteristically shifted *ca* 5 ppm downfield when one of the C-4 geminal methyl groups is replaced by a prenyl chain (δ 48.8-49.8) as in **7**, **9**, **10**, **11**, **15** and **16** (Jayasuriya et al. 1991; Rocha et al. 1996). A further downfield shift *ca* 13 ppm can be observed when both of the two C-4 geminal methyl groups are replaced by a prenyl and an *iso*-butyryl chain (δ 57.5), as in **13** (Rocha et al. 1996).

Characteristic substituted aromatic carbon signals, C-5' (δ 154.6-166.1), C-6' (δ 103.7-107.5), C-7' (δ 158.2-163.4), C-8' (δ 103.6-108.3), C-9' (δ 154.6-163.3), C-10' (δ 101.7-111.5) present on the phloroglucinol moiety are often reported as interchangeable values between C-6', C-8' and C-10', and between C-5', C-7' and C-9'. Additionally, the aromatic non-substituted C-10'carbon signal is displayed at further upfield as in **10** (δ 93.4) and **23** (δ 94.2).

The benzopyran ring skeleton with a 2,2-dimethyl chromene system displays a characteristic dimethyl-substituted C-2' quaternary carbon signal (δ 77.9-81.5), two olefinic carbon signals C-3' and C-4' (δ 123.9-126.4; δ 117.0-117.5), in addition to two geminal methyl signals (δ 27.5-28.2). In the isoprenylmethyl-methyl-chromene system, an additional methylene carbon signal (δ 36.5-41.8) appears as in **25**, **26** and **27**. In this chromene system, the olefinic carbon signals C-3' and C-4', as present in **25** (δ 123.1; δ 123.7), can be absent and replaced by

adjacent C-3' methine (δ 66.7) and C-4' methylene (δ 26.2) carbon signals, indicating the presence of a hydroxyl group at C-3'as in **26** and **27**.

Signals from carbons in the C-prenyl side chains are recognizable as a set of a methylene carbon, two vinyl carbon, and two olefinic methyl carbon signals. The C-prenyl side chain is distinguishable when attached to the C-4 of the filicinic acid moiety (e.g. in **7**, C-1'', δ 38.9; C-2'', δ 118.2; C-3'', δ 136.5; 3''-Me, δ 17.6, δ 25.6); to the C-10' of the phloroglucinol moiety (e.g. in **11**, C-4', δ 22.3; C-3', δ 123.9; C-2', δ 132.7; C-2'-Me, δ 18.0, δ 25.9); and when forming part of the isoprenylmethyl-methyl-chromene system as in **25**, **26** and **27** (**Table 6**). Signals of the O-prenyl side chain attached to the C-5'/C-9' of the phloroglucinol moiety are reported in **10** (C-4', δ 67.1; C-3', δ 118.1; C-2', δ 142.5; C-2'-Me, δ 18.2, δ 25.9) and **23** (**Table 6**).

Less frequently observed carbon signals include those from a mono substituted benzene ring (C-13'-C17', δ 127.3-132.0) as seen in **23** and **24**, and the signals of a C-geranyl side chain that are revealed as a set of three geminal-methyl, three methylene and four vinyl carbon signals as in **22** and **24** (**Table 6**).

X-ray crystallography

The crystallographic data reported for **3**, **14**, **16** (Leal et al. 2010), the bromo derivate of **16** (Parker et al. 1968), **18** (Rocha et al. 1995), **23** (Ishiguro et al. 1985) and **25** (Hu et al. 2000) reveals unequivocally that these compounds are composed of a filicinic acid and an acylphloroglucinol moieties linked by a methylene bridge held in a rigid conformation by two hydrogen bonds [e.g. in **14**, O(5')-H---O(1) and O(3)-H---O(7')]. Two other hydrogen bonds with unusually short oxygen-oxygen distances occur in these molecules [e.g. in **14**, O(5)-H---O(8) and O(7')H---O(11')] (Leal et al. 2010), and these correlate with very low chemical shifts for the chelated protons in the ^{1}H-NMR spectra.

Tautomerism

In general, acylphloroglucinols are constituted of tautomeric mixtures, which more or less rapidly interconvert. In solution they usually exist in one major tautomeric form, but the

tautomeric composition depends on the effect of pH, solvent polarity, and temperature (Verotta 2002; Katritzky et al. 2010). The observed chemical isomerism can complicate NMR spectra interpretation; however, some strategies have been effectively used to overcome this difficulty.

For example, when dissolved in $CDCl_3$, hyperforin shows primarily broad ^1H NMR signals and a poorly resolved ^{13}C NMR spectrum that denotes that hyperforin exists in a tautomeric mixture, derived from the keto-enol equilibrium of its β-dicarbonyl skeleton (Cui et al. 2004; Hostettmann and Wolfender 2005). These facts, in addition to the poor stability of hyperforin in $CDCl_3$, have led to the use of CD_3OD and acetone-d_6 as NMR solvents (Mannila et al. 2002; Cui et al. 2004). Enolic protons signals (observed in $CDCl_3$ at 7.00–7.50 ppm) are not observed in CD_3OD or acetone-d_6 because of the solvent interaction with the enolic hydroxyl group of hyperforin. Specially, this interaction seems to block the structure of hyperforin to favor a certain tautomeric form, which explains the sharper peaks observed in acetone-d_6 and CD_3OD than in $CDCl_3$ (Mannila et al. 2002). To further study the effect of tautomerization on the ^1H NMR spectrum of hyperforin, two derivatization reactions were reported using chlordimethylether (CH_3OCH_2Cl) and benzoyl chloride (C_6H_4Cl) to convert the enolic hydroxyl group to its respective derivatives. The ^1H NMR spectra of both derivatized hyperforin forms (in $CDCl_3$) showed peaks clearly sharper than those obtained from the same sample before the derivatization, further unveiling the tautomeric nature of hyperforin (Mannila et al. 2002).

In the same way, both the ^1H and the ^{13}C NMR spectra of many dimeric acylphloroglucinol compounds are reported as showing certain anomalies, which can best be explained by the expected keto-enol tautomerization of the filicinic acid moiety. The possible tautomeric structures of the acylfilicinic acid unit are reported by Äyräs et al. (1981). This tautomerization complicates the spectral interpretation, but in no case prohibits complete assignments in the ^1H and ^{13}C NMR spectra (Äyräs et al. 1981; Ishiguro et al. 1985; Jayasuriya et al. 1994). Additionally, it is interesting to notice that many unclear elements can be solved by carefully analysis of proton-coupled ^{13}C NMR spectra (Äyräs et al. 1981). Initial observations in the ^1H NMR spectra of **14** showed that the singlet at δ 1.46 ppm corresponding to the geminal dimethyl groups of the acyl filicinic moiety (accounted for, in part, by two geminal methyl groups of the chromene system) has a shoulder on the low-field

side that sharpens into a separate peak on warming or cooling. This temperature dependence was more clearly seen in dihydrouliginosin B, because the peak in question, now at δ 1.50 ppm (the geminal dimethyl groups of the chromene system appear at δ 1.38 ppm), is a singlet at room and above temperature, but a doublet at low temperature, thus revealing the tautomeric nature of **14** and its dihydro derivative (Parker and Johnson 1968). In addition to the characteristic singlets in low field due to the hydroxyl groups in the ^{1}H NMR spectrum of many dimeric acylphloroglucinols, additional minor signals (e.g. downfield region in the ^{1}H NMR spectra of **14** and **16** in **Fig. 3**) due to a minor tautomeric components can be observed (Äyräs et al. 1981). Other noteworthy features are the very broad signals observed for protons within the acyl side chains (9-Me_2, 12'-H and 13'-Me) in the ^{1}H NMR spectrum of **20** measured at 360 MHz, while the spectrum at 200 MHz showed all explicit signals (Ishiguro et al. 1987). These observations suggest that **20** exists in wide range of tautomeric structures that come from the keto-enol equilibrium in the 1,3-dicarbonyl system, that coincides with the low field-shifted signals for C-3 and C-5 in the ^{13}C NMR spectrum of **20** (Ishiguro et al. 1987) and most dimeric acylphloroglucinols.

The keto-enol tautomerism has been also observed in many benzophenone derivatives such as the guttiferones and related compounds (spectra in $CDCl_3$ show tautomeric pairs), for which ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD + 0.1% trifluoroacetic acid (TFA). This acidic medium enhances the rate of the keto-enol interconversion of the β-hydroxy-α,β-unsaturated ketone, thus simplifying the spectral analysis (Gustafson et al. 1992; Hamed et al. 2007). The use of pyridine-d_5 to reduce signal overlap has been also explored for the structure elucidation of some guttiferones (Williams et al. 2003; Cao et al. 2007). Another approach to obtain more highly defined NMR signals of acylphloroglucinols showing keto-enol tautomerism includes the acetylation of all hydroxyl groups of the molecule, thus blocking the potential interconversion among tautomers as reported in the structure elucidation of elaphopilosin A, a fern dimeric acylphloroglucinol (Socolsky et al. 2009).

Analysis in vegetal matrices

Thin layer chromatography (TLC)

Overall contents of dimeric acylphloroglucinols are usually reported to be lower compared to flavonoids in *Hypericum* species from *Brathys* and *Trigynobrathys* sections (Barros et al. 2013); therefore TLC analysis of dimeric acylphloroglucinols are usually carried out on enriched lipophilic fractions rather than on crude alcoholic extracts. Mobile phases commonly used are composed of mixtures of *n*-hexane/chloroform, *n*-hexane/dichloromethane (50:50), *n*-hexane/ethyl acetate (90:10), *n*-hexane/ethyl ether (90:10), acidified or not with 0.25% formic acid. On TLC, dimeric acylphloroglucinols are readily identified by their characteristic UV absorbance at 254 and 356 nm. Characteristic reddish-orange or yellow spots are observed after spraying the TLC plate with Godin or anisaldehyde-sulfuric acid reagent (recipes can be found in Hostettmann and Marston 1995, and Wagner and Blatt 1996).

High performance liquid chromatography (HPLC)

An HPLC-UV method is reported by Nunes et al. 2009 for the analysis of some dimeric acylphloroglucinols in lipophilic extracts of *H. polyanthemum* and other species from section *Brathys* and *Trigynobrathys* (Barros et al. 2013). In this method, separations are carried out in a Waters Nova-Pack C18 column (4 μm, 3.9 x 150 mm) using an isocratic mobile phase program (95% acetonitrile, 5% water, 0.01% TFA), with UV detection at 220 nm. This methodology was also applied for the analysis of dimeric acylphloroglucinols **3**, **14** and **15** in supercritical CO_2 extracts (Cargnin et al. 2010; Barros et al. 2011; Almeida et al. 2013).

Over the last years, many HPLC methods have been developed to achieve the analysis of crude extracts and preparations of *H. perforatum* (Meier 2003). Since these extracts/preparations usually contain at least six major chemical classes, including naphthodianthrones (hypericin and pseudohypericin), flavonoids (rutin, hyperoside, isoquercitrin, quercitrin and quercetin), phloroglucinols (hyperforin and adhyperforin), biflavonoids (I3, II8-biapigenin, I3′, II8-biapigenin), proanthocyanidins and phenolic acids (chlorogenic and caffeic acid), HPLC methods were developed in the attempt to achieve good separations in a reasonable total run time that were previously carried out in more than 50 min (Liu et al. 2000; Li and Fitzloff 2001). One of the most feasible methods for this purpose is that reported by Ganzera et al. (2002), in which separations are carried out in a Synergi MAX-RP 80 Å column (4μm, 4.6 mm x 150 mm) using 10 mm ammonium acetate buffer (solvent A), equilibrated to pH 5.0 with glacial acetic acid, and acetonitrile/methanol (9:1; solvent B)

as mobile phase in a gradient system program (from 87:13, A:B to 83:17 in 10 min, then to 0:100 in 25 min), with a total run time of 35 min, and 10 min of equilibration time. Detection is performed at 270 nm, peak purity determined by analysis of DAD spectra, and confirmation of peak identity by MS.

This latter method has been used for characterizing the metabolic fingerprint of a crude methanol extract of *H. gentianoides*, in which chlorogenic acid, hyperoside, isoquercitrin, quercitrin, and quercetin were detected along with at least nine metabolites (retention time from 30 to 45 min) with distinctive three-peaked UV spectra and absorption maxima of 220, 300, and 350 nm, or of 226, 287, and 357 nm (Hillwig et al. 2008). Three of these compounds were identified as **12**, **13** and **19** by 2-D NMR (Hillwig 2008). Further HPLC-MS and MS/MS analyses of the distinctive fragmentation pattern of dimeric acylphloroglucinols led to the identification of **14**, **29**, **30**, **31** and a monomeric phlorisobutyrophenone derivative (Crispin et al. 2013).

Dimeric acylphloroglucinols in Peruvian *Hypericum* species

Background

Most *Hypericum* species native to the high mountain regions in South America belong to the taxonomic section *Brathys* and, to a lesser extent, *Trigynobrathys*. In contrast to the widely used *H. perforatum*, the species of the Páramos regions of Central and South America have been rarely phytochemically examined (Crockett et al. 2010). Nevertheless, several studies of species belonging to sections *Brathys* and *Trigynobrathys* occurring at lower elevations have been published. These include reports on the isolation of flavonoids (Rocha et al. 1995), xanthones (Rocha et al. 1994), phloroglucinol derivatives, (Rocha et al. 1996), and less frequently, benzopyrans (Ferraz et al. 2001) and benzophenones (Bernardi et al. 2005; Crockett and Robson 2011), while naphthodianthrones were not detected in almost 15 investigated species from these two sections (Ferraz et al. 2002; Crockett et al. 2005; Nuevas-Paz et al. 2005). Since dimeric acylphloroglucinols are commonly reported in *Hypericum* species from sections *Brathys* and *Trigynobrathys*, it is proposed that these compounds could be considered chemotaxonomic markers for the species of these two sections (Nör et al. 2004;

Barros et al. 2013). This proposition is here further explored with the examples of some Peruvian *Hypericum* species.

Experimental

General procedures

Formic acid (Vetec, Rio de Janeiro, Brazil), acetone, dichloromethane, ethyl acetate and *n*-hexane (F. Maia, Cotia, Sao Paulo, Brazil), all reagent grade, were regularly used during the extraction and isolation procedures. In order to best visualize the isolation development, the extracts and fractions were submitted to TLC using precoated silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) as a stationary phase using three different mobile phases composed of *n*-hexane/dichloromethane (50:50 v/v), *n*-hexane/ethyl acetate (90:10 v/v) and *n*-hexane/ethyl acetate (95:5 v/v) with 0.25% formic acid as mobile phases. After elution, TLC plates were observed under 254 and 356 nm UV light and further examined after spraying with anisaldehyde-sulfuric acid reagents.

1D- (^{1}H and ^{13}C) and 2D- (HSQC, HMBC, COSY) NMR experiments were performed on a 400 MHz Varian MR400 spectrometer. Compounds **12**, **14** and **16** were dissolved in $CDCl_3$, with tetramethylsilane (TMS) as internal standard; while **15** and **17** were dissolved in acetone-d_6, spectra referenced against residual non deuterated solvent.

Plant material

Hypericum andinum Gleason (Amparaes, Cuzco, Peru, 3432 m elevation; Ccana-Ccapatinta 06), *H. laricifolium* Juss (Cumbemayo, Cajamarca, Peru, 3450 m elevation; Ccana-Ccapatinta 08) (Shrubs, *Brathys*), *H. brevistylum* Choisy (Paqchaq, Cuzco, Peru, 3573 m elevation; Ccana-Ccapatinta 04) and *H. silenoides* Juss (Pumahuanca, Cuzco, Peru, 3189 m elevation; Ccana-Ccapatinta 02) (herbs, *Trigynobrathys*) are species that occur in high altitude Páramo habitats. The aerial parts of these plants were collected (under consent of Dirección General Forestal y de Fauna Silvestre of the Republic of Peru, 0147-2010-AG-DGFFS-DGEFFS) and identified by Botanist MsC. Washington H. Galiano Sánchez (Principal Professor of the academic department of Biology, UNSAAC). Voucher specimens were deposited in the

Herbarium of the Federal University of Rio Grande do Sul (ICN), Brazil and in the Herbarium Vargas (CUZ) of the Universidad Nacional de San Antonio Abad del Cusco - UNSAAC, Peru.

Extraction and isolation

Air dried and powdered vegetal material (*H. andinum*, 1000 g*; H. brevistylum*, 850 g; *H. laricifolium*, 400 g; *H. silenoides*, 1100 g) was successively extracted by maceration with *n*-hexane over 72 hours (5 times, plant-solvent ratio 1:5). The extracts were evaporated to dryness under reduced pressure and then treated with cold acetone to obtain acetone-soluble fractions (ASF) (18.5, 24.5, 12.2 and 27.7 g for *H. andinum*, *H. brevistylum*, *H. laricifolium* and *H. silenoides*, respectively) and insoluble fatty residues for each.

The ASF of *H. laricifolium* (12.2 g) was subjected to silica gel CC using a gradient elution of *n*-hexane/DCM 100:0-0:100 to afford 12 fractions (Fr. 1-12). Fr. 2-3 were subjected to repeated CC using a gradient elution of *n*-hexane/EtOAc 100:0-90:10 to afford 60 mg of **15** (eluted with 97:3 *n*-hexane/EtOAc) and 20 mg of **17** (eluted with 93:7 *n*-hexane/EtOAc). Fr. 6-7 were subjected to CC using a gradient elution of *n*-hexane/EtOAc 100:0-90:10 to afford 80 mg of **14** (eluted with 95:0 *n*-hexane/EtOAc) and 30 mg of **16** (eluted with 90:10 *n*-hexane/EtOAc). The ASF of *H. silenoides* (27.7 g) was subjected to silica gel CC using a gradient elution *n*-hexane/DCM 100:0-0:100 to afford 10 fractions (Fr. 1-10). Fr. 2-4 were subjected to CC with *n*-hexane/EtOAc 100:0-90:10 to afford 90 mg of **14** (eluted with 95:0 *n*-hexane/EtOAc) and 15 mg of **16** (eluted with 90:10 *n*-hexane/EtOAc). Fr. 8 was subjected to repeated CC with *n*-hexane/EtOAc 100:0-50:50 acidified with 0.25% HCOOH to afford 30 mg of **12** (eluted with 70:30 *n*-hexane/EtOEt with 0.25% HCOOH). The ASF of *H. andinum* and *H. brevistylum* were also submitted to isolation procedures similar to that for *H. laricifolium* and *H. silenoides*; **14**, **16** and **12** were isolated from both species.

Discussion

Dimeric acylphloroglucinols derivatives, hyperbrasilol B (**15**), isohyperbrasilol B (**17**), uliginosin B (**14**), isouliginosin B (**16**), and uliginosin A (**12**) were identified after the analysis of their ^{1}H and ^{13}C NMR, COSY, HSQC and HMBC spectra and by comparison with

reported data. Compounds **15** and **17** have been previously isolated from the aerial parts of *H. brasiliense* (Rocha et al. 1996). Compound **17** has been also described in *H. caprifoliatum*, *H. connatum* and *H. laricifolium* by HPLC detection (Barros et al. 2013). Hence, the isolation of **15** and **17** in the aerial parts of *H. laricifolium* is reported. Compound **14** was firs isolated from aerial parts of *H. uliginosum* (Taylor and Brooker 1969) and has been described from almost eighteen *Hypericum* species from sections *Brathys* and *Trigynobrathys* (**Table 1**). Compounds **14** and **16** were isolated from all the four Peruvian *Hypericum* species explored. However, initially described as a by-product in the synthesis of **14** (Meikle and Stevens 1978), **16** was originally described as naturally occurring in the aerial parts of *H. brasiliense* (Rocha et al. 1995). Moreover, compound **12** was first isolated from *H. uliginosum* (Taylor and Brooker 1969), further characterized in *H. brasiliense* (Rocha et al. 1995), recently identified in *H. gentianoides* (Hillwig 2008; Crispin et al. 2013), and here isolated from *H. andinum*, *H. brevistylum* and *H. silenoides*.

Valine, and possibly pyruvate, are likely primary metabolite precursors of acylphloroglucinols in *H. perforatum* (Karppinen et al. 2007), that serve as plausible templates for the initial steps of acylphloroglucinol biosynthesis in *Hypericum* spp. Based on the "monomer pool concept" postulated for the biosynthesis of dimeric acylphloroglucinols in *H. gentianoides* (Crispin et al. 2013), a possible biosynthetic route to dimeric acylphloroglucinols in *H. andinum*, *H. brevistylum*, *H. laricifolium* and *H. silenoides* is proposed in **Fig. 4**. The monomer pool concept is based on a) the structure characterization of nine prevalent acylphloroglucinols in *H. gentianoides*, that included one monomeric phlorisobutyrophenone (PIB) derivative and eight dimeric acylphloroglucinols, based on LC/ESI-MS and Q-TOF mass spectrometry, and b) the identification of eight additional PIB derivatives, in trace amount by Q-TOF spectrometry, likely to be precursors of the major compounds observed, thus supporting the proposition that dimeric acylphloroglucinols are synthesized via modification of PIB to yield diverse phloroglucinol and filicinic acids moieties, followed by dimerization of a phloroglucinol and a filicinic acid monomer to yield the observed dimeric acylphloroglucinols (Crispin et al. 2013). The enzymatic formation of PIB and its prenylated derivative by an isobutyrophenone synthase and phlorisobutyrophenone dimethylallyltransferase, respectively, have been demonstrated using cell-free extracts from cell cultures of *H. calycinum* (Boubakir et al. 2005; Klingauf et al. 2005). The enzymes involved in the creation of a methylene bridge in dimeric acylphloroglucinols remain unknown. However the mechanism for EC 1.21.3.3,

which forms a methylene bridge between two phenolic rings has been described (Kutchan and Dittrich 1995), and applied to propose the methylene bridge formation in dimeric acylphloroglucinol biosynthesis (Crispin et al. 2013).

Dimeric acylphloroglucinols have awakened academic interest in view of the wide array of bioactivities they display. Extensive pharmacological work has shown that **14** has a promising analgesic and antidepressant profile. It induced an antinociceptive effect when administered at 15 mg/kg, i.p., as measured by the hot-plate and abdominal writhing tests; it also reduced the immobility time in the mouse forced swimming test at 10 mg/kg, p.o. (Stein et al. 2012; Stolz et al. 2012). Similarly, **15** has been recently shown to possess antinociceptive activity in the hot-plate test when administered to mice, with a maximal effect at 13 mg/kg, p.o. (Sakamoto et al. 2012). Additionally, the ability of **12** to inhibit the release of lipopolysaccharides (LPS)-induced inflammatory mediators in RAW 264.7 mouse macrophages has been demonstrated at 2.6, 2.0 and 0.6 μM concentrations (Huang et al. 2011). Among other recently described bioactivities for **14** are the anti-*Trichomonas vaginalis* activity (Cargnin et al. 2013) and potential antiproliferative activity against OVCAR-3-human ovarian carcinoma cells (Pinhatti et al. 2013).

Chemosystematic significance

Dimeric acylphloroglucinols are usually described in *Hypericum* species from sections *Brathys* and *Trigynobrathys.* However, their occurrence is not exclusive to *Hypericum,* but is extended to other genera such as *Aspidium, Dryopteris*, *Elaphoglossum*, *Eucalyptus*, *Helichrysum*, *Myrtus*, and *Mallotus* (Singh and Bharate 2006; Socolsky et al. 2012b). Nevertheless, of the thirty-one dimeric acylphloroglucinols described for *Hypericum* (**Table 1**), only three of them are known for other genera (**1**, **2** and **3**), thus demonstrating that *Hypericum* displays a specialized chemical diversity.

Several surveys have explored the chemotaxonomic significance of naphthodianthrones (Kitanov 2001), flavonoids (Cirak et al. 2010) and acylphloroglucinols (Nör et al. 2004; Barros et al. 2013) among the species and taxonomic sections of *Hypericum*. Focusing on acylphloroglucinol derivatives, as a general trend it has been shown that some sections seem to produce mostly monocyclic and bicyclic prenylated phloroglucinol derivatives (sections

grouped as G1 by Crockett 2012), while tricyclic phloroglucinol derivatives may predominate in others (sections grouped as G2 by Crockett 2012), and dimeric acylphloroglucinols dominate in section *Brathys* and *Trigynobrathys* (Crockett 2012; Barros et al. 2013), highlighting the utility of acylphloroglucinol derivatives as potential chemotaxonomic markers. However, representatives of less than a third of the taxonomic sections of *Hypericum* have been examined for acylphloroglucinols and related compounds (Crockett and Robson 2011), thus firm conclusions can not yet be made regarding their utility as chemotaxonomic markers (Crockett and Robson 2011; Crockett 2012).

Nevertheless, to the best of our knowledge, dimeric acylphloroglucinols are exclusively distributed in sections *Brathys* and *Trigynobrathys* within *Hypericum*; therefore we hypothesize that these compounds have a chemotaxonomic utility at the sectional or subsectional level (Barros et al. 2013). The isolation of dimeric acylphloroglucinols in four Peruvian *Hypericum* species further supports the proposition that these compounds could be considered as chemotaxonomic markers for the section *Brathys* and *Trigynobrathys* in *Hypericum*.

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Figure captions

Fig. 1 Dimeric acylphloroglucinols identified in *Hypericum* species from sections *Brathys* and *Trigynobrathys*. Note that in many cases the carbon skeleton numbering scheme were reassigned differently from their original sources following arbitrary criteria

Fig. 2 Primary fragmentation and major fragments expected in drummondin C

Fig. 3 Selected region of the HMBC spectra of uliginosin B and isouliginosin B recorded in $CDCl_3$ and principal correlations involving the methylene bridge, hydroxyl groups and olefinic protons from the benzopyran ring

Fig. 4 Proposed biosynthetic pathway of dimeric acylphloroglucinols in *Hypericum andinum*, *H. brevistylum*, *H. laricifolium* and *H. silenoides*. Valine and possibly pyruvate are likely primary metabolite precursors of acylphloroglucinols in *H. perforatum* (Karppinen et al. 2007). The enzymatic formation of PIB and 3'prenPIB by an isobutyrophenone synthase and phlorisobutyrophenone dimethylallyltransferase is reported in *H. calycinum* (Boubakir et al. 2005; Klingauf et al. 2005). The methylene bridge formation mechanism is proposed elsewhere (Crispin et al. 2013) based on the mechanism of EC 1.21.3.3. PIB and PIB derivatives, 8ib22meC57diol, 3'prenPIB and 3'3'me6'oxoPIB are reported in *H. gentianoides* (Crispin et al. 2013). 6ib22meC57diol and 3'pren3'me6oxoPIB are PIB derivatives here hypothetically proposed. Abbreviations: PIB, phlorisobutyrophenone; 6ib22meC57diol, 6-isobutyryl-2,2-dimethyl-chromene-5,7-diol; 8ib22meC57diol, 8-isobutyryl-2,2-dimethyl-chromene-5,7-diol; 3'prenPIB, 3'-prenyl-phlorisobutyrophenone; 3'3'me6'oxoPIB, 3',3'-dimethyl-6'-oxo-phlorisobutyrophenone; 3'pren3'me6oxoPIB, 3'-prenyl,3'-methyl-6'-oxo-phlorisobutyrophenone

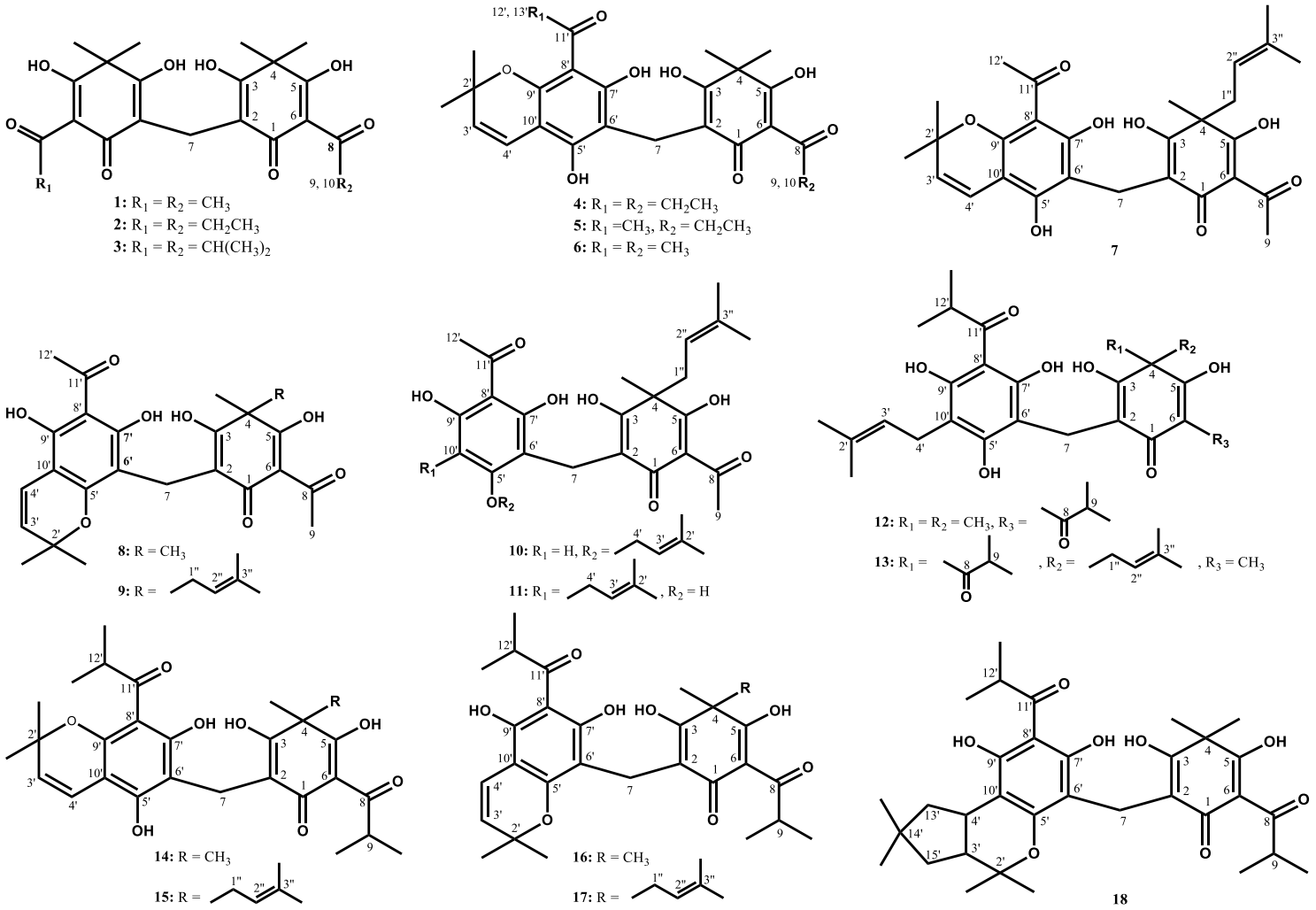
1: R1 = R2 = CH3
2: R1 = R2 = CH2CH3
3: R1 = R2 = CH(CH3)2
4: R1 = R2 = CH2CH3
5: R1 =CH3, R2 = CH2CH3
6: R1 = R2 = CH3
7
8: R = CH3
9: R =
10: R1 = H, R2 =
11: R1 = , R2 = H
12: R1 = R2 = CH3, R3 =
13: R1 = , R2 = , R3 = CH3
14: R = CH3
15: R =
16: R = CH3
17: R =
18

Fig. 1

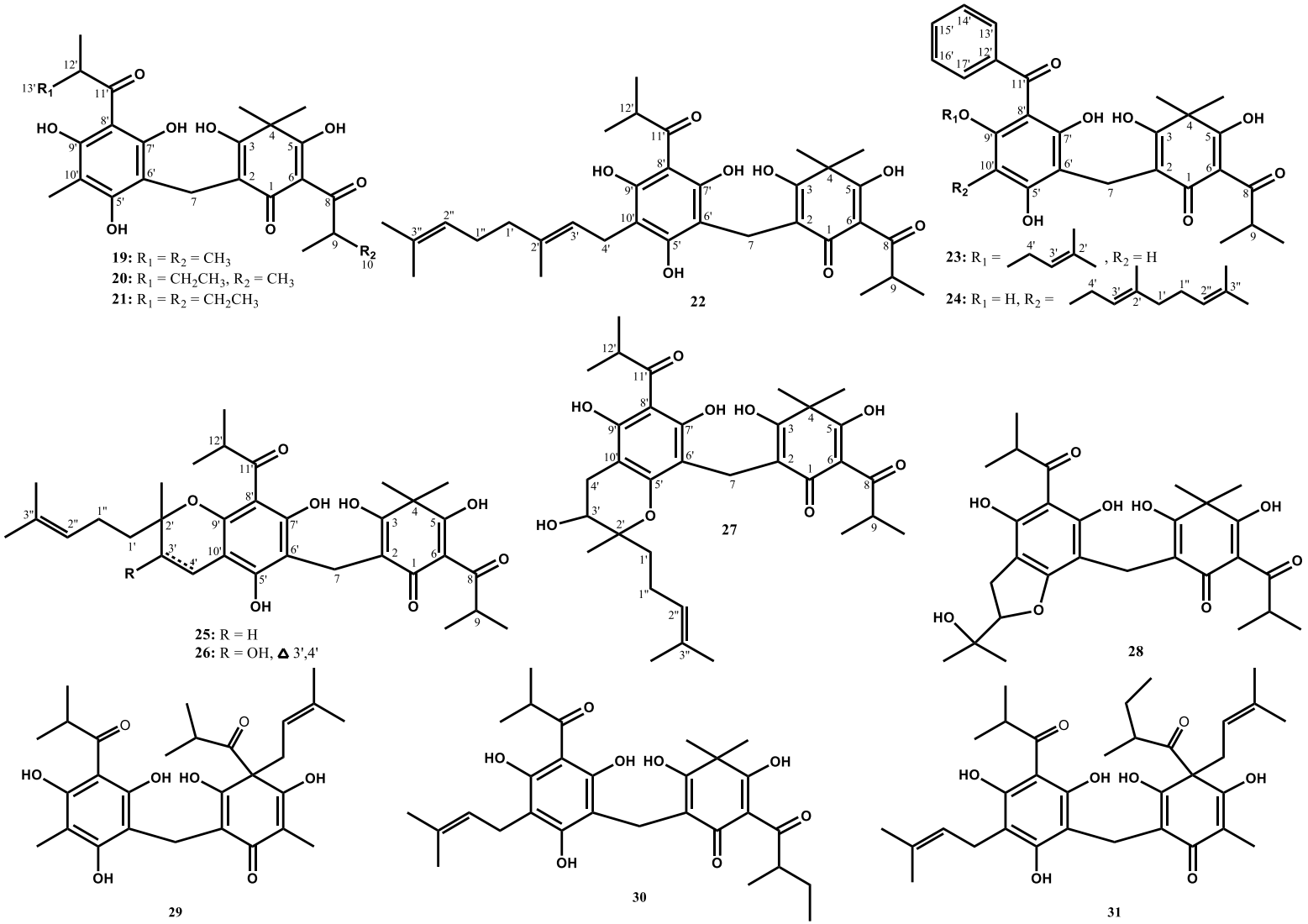

Fig. 1 (Continued)

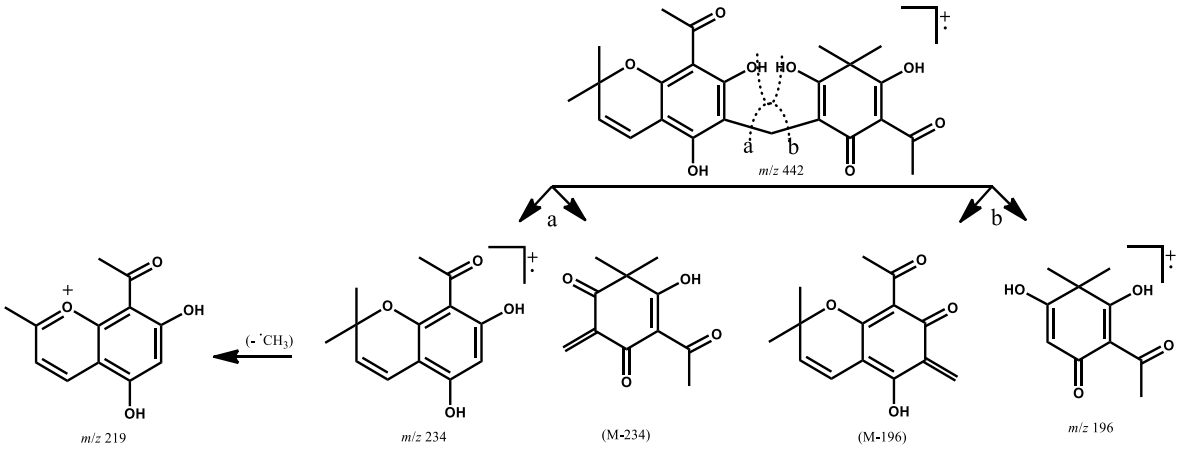
m/z 442
a
b
(- ·CH3)
m/z 219
m/z 234
(M-234)
(M-196)
m/z 196

Fig. 2

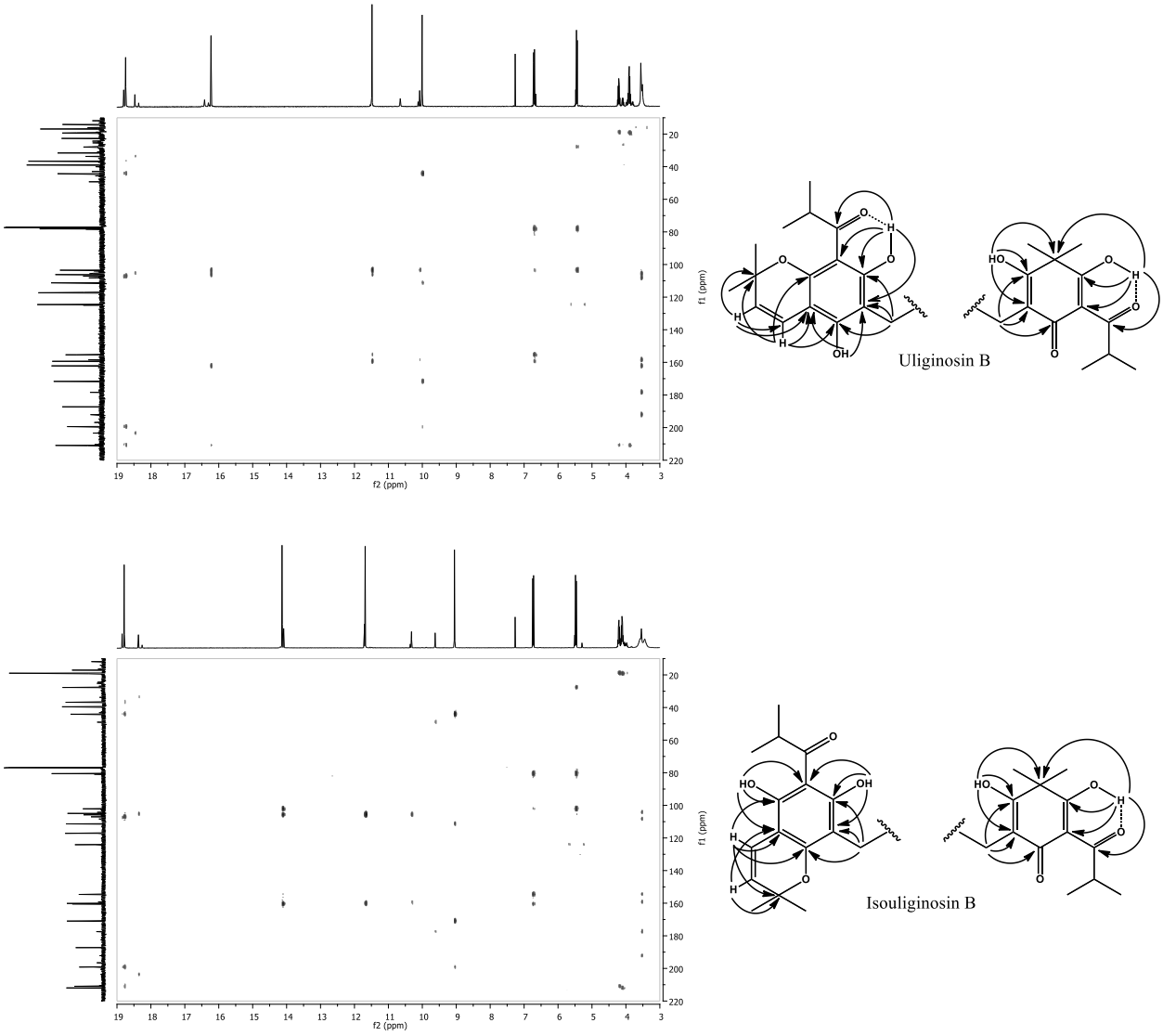
f2 (ppm)
f1 (ppm)
Uliginosin B
Isouliginosin B

Fig. 3

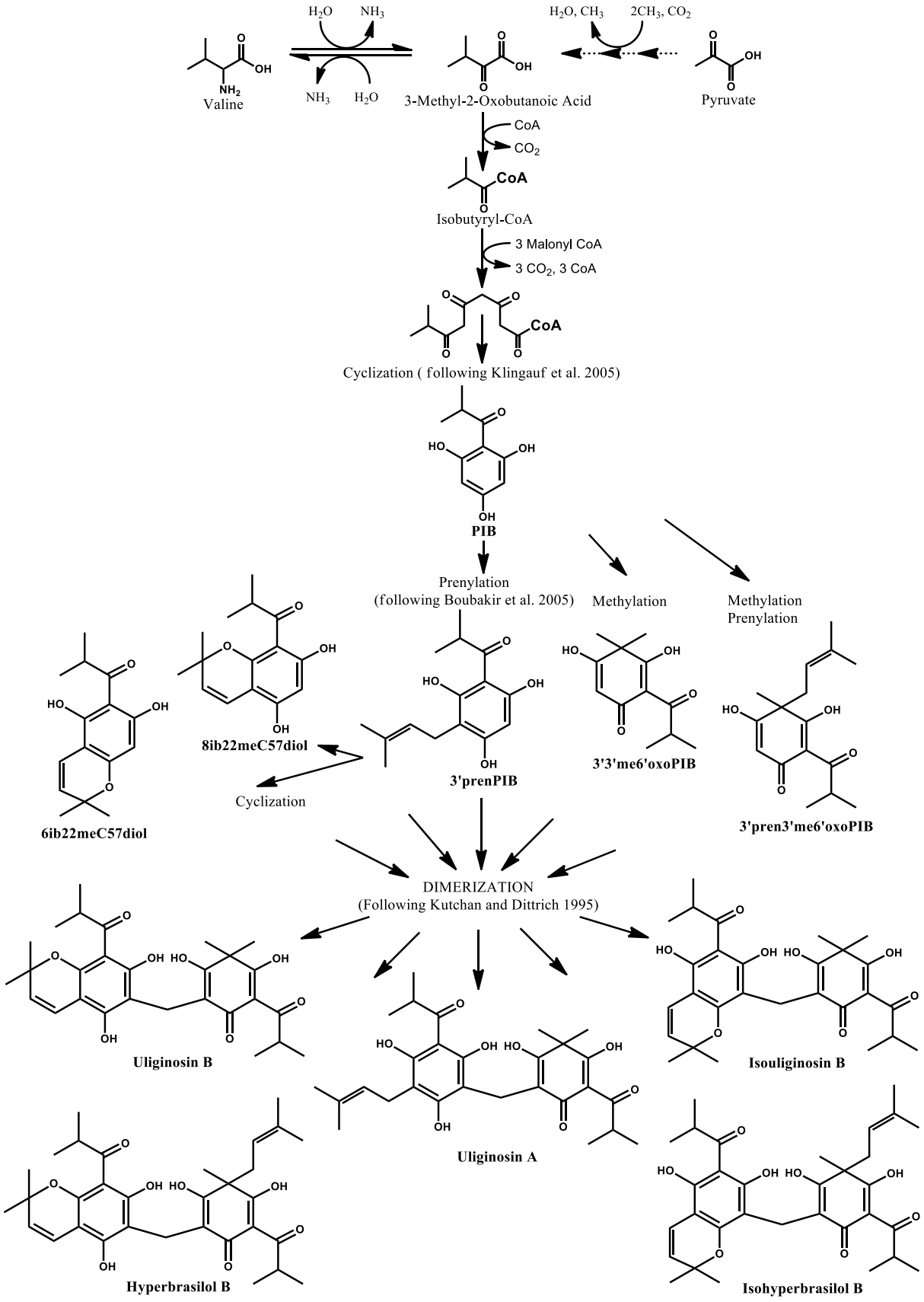
Valine
3-Methyl-2-Oxobutanoic Acid
Pyruvate
Isobutyryl-CoA
Cyclization (following Klingauf et al. 2005)
PIB
Prenylation
(following Boubakir et al. 2005)
Methylation
Methylation
Prenylation
8ib22meC57diol
3'prenPIB
3'3'me6'oxoPIB
3'pren3'me6'oxoPIB
6ib22meC57diol
Cyclization
DIMERIZATION
(Following Kutchan and Dittrich 1995)
Uliginosin B
Uliginosin A
Isouliginosin B
Hyperbrasilol B
Isohyperbrasilol B

Fig. 4

Table 1 UV, EI-MS data and sources of dimeric acylphloroglucinols in *Hypericum* species from *Brathys* and *Trigynobrathys* sections

Compound	UV λ_{max} nm	EI-MS *m/z*	Sources in *Hypericum**	Reference
1 Albaspidin AA	λ^{EtOH} 336, 339, 294	404[M^+], 209, 208, 196, 193, 181, 168, 153, 137	*Brathys*: *H. drummondii*	Jayasuriya et al. 1989
2 Albaspidin PP	λ^{EtOH} 344, 294, 264	432[M^+], 417, 223, 210, 195, 193, 182, 167	*Brathys*: *H. drummondii*	Jayasuriya et al. 1989
3 Albaspidin iBiB (Japonicin A)	λ^{MeOH} 341, 221	460[M^+], 445, 417, 235, 209, 182, 165, 69	*Trigynobrathys: H. brasiliense, H. campestre, H. caprifoliatum, H. connatum, H. japonicum, H. linoides, H. myrianthum, H. polyanthemum, H. salvadorense*	Rocha et al. 1995 Bernardi 2007 Barros et al. 2013
4 Drummondin A	λ^{EtOH} 358, 258	470[M^+], 455, 261, 245, 233, 227, 215	*Brathys*: *H. drummondii*	Jayasuriya et al. 1989
5 Drummondin B	λ^{EtOH} 356, 282, sh 270	456[M^+], 441, 247, 234, 233, 231, 219, 213	*Brathys*: *H. drummondii*	Jayasuriya et al. 1989
6 Drummondin C	λ^{EtOH} 355, 284	442[M^+], 427, 247, 234, 231, 219, 213, 201	*Brathys*: *H. drummondii*	Jayasuriya et al. 1989
7 Drummondin D	λ^{MeOH} 356, 287	496[M^+], 428, 409, 247, 231, 219	*Brathys*: *H. drummondii*	Jayasuriya et al. 1991
8 Isodrummondin C		442[M^+]	*Brathys*: *H. drummondii*	Jayasuriya et al. 1994
9 Isodrummondin D	λ^{MeOH} 357, 314, 278	496[M^+], 428, 409, 246, 231, 219, 201	*Brathys*: *H. drummondii*	Jayasuriya et al. 1991
10 Drummondin E	λ^{MeOH} 354, 294	498[M^+], 343, 247, 236, 193, 181	*Brathys*: *H. drummondii*	Jayasuriya et al. 1991
11 Drummondin F	λ^{MeOH} 350, 300	498[M^+], 355, 236, 195, 193, 182, 165	*Brathys*: *H. drummondii*	Jayasuriya et al. 1991
12 Uliginosin A	λ^{Hexane} 290, 230 $\lambda^{Cyclohexane}$ 293, 229	500[M^+], 457, 445, 401, 277, 264, 221, 209, 165, 153, 121, 69	*Brathys: H. andinum**, H. gentianoides* *Trigynobrathys: H. brasiliense, H. brevistylum**, H. mutilum, H. silenoides**, H. uliginosum*	Schühly et al. 2007 Hillwig 2008 Barros et al. 2013
13 Hyperbrasilol C	λ^{MeOH} 348, 301, 224	554[M^+], 467, 277, 264, 221, 209, 165, 69	*Brathys: H. gentianoides* *Trigynobrathys: H. brasiliense*	Rocha et al. 1995 Hillwig 2008
14 Uliginosin B	$\lambda^{Cyclohexane}$ 270, 230	498[M^+]	*Brathys: H. andinum**, H. gentianoides, H. laricifolium**,* *Trigynobrathys: H. brasiliense, H. brevistylum**, H. campestre, H. caprifoliatum, H. carinatum, H. connatum, H. japonicum, H. linoides, H. myrianthum, H. mutilum, H. polyanthemum, H. salvadorense, H. silenoides**, H. ternum, H. uliginosum*	Taylor and Brooker 1969 Ishiguro et al. 1986 Schühly et al. 2007 Barros et al. 2013 Crispin et al. 2013
15 Hyperbrasilol B	λ^{MeOH} 357, 287, 226	552[M^+], 465, 275, 259, 247, 167, 69	*Brathys: H. laricifolium*** *Trigynobrathys: H. brasiliense, H. caprifoliatum, H. connatum*	Rocha et al. 1996 Barros et al. 2013
16 Isouliginosin B	λ^{MeOH} 355, 315, 277, 224	498[M^+], 483, 455, 275, 262, 247, 219	*Brathys: H. andinum**, H. laricifolium*** *Trigynobrathys: H. brasiliense, H. brevistylum**, H. silenoides***	Rocha et al. 1995
17 Isohyperbrasilol B	λ^{MeOH} 357, 279, 216	552[M^+], 484, 465, 275, 259, 247, 167, 69	*Brathys: H. laricifolium*** *Trigynobrathys: H. brasiliense*	Rocha et al. 1996
18 Hyperbrasilol A	λ^{MeOH} 355, 306, sh 224, 209	568[M^+], 332, 289, 209, 193, 179, 165, 150, 111, 95, 71, 69	*Trigynobrathys: H. brasiliense*	Rocha et al. 1996
19 Saroaspidin A	λ^{MeOH} 350, 302, 225	446[M^+], 280, 224, 187, 167	*Brathys: H. gentianoides* *Trigynobrathys: H. japonicum*	Ishiguro et al. 1987 Hillwig 2008
20 Saroaspidin B	λ^{MeOH} 348, 302, 225	460[M^+], 236, 224, 209, 181, 167	*Trigynobrathys: H. japonicum*	Ishiguro et al. 1987
21 Saroaspidin C	λ^{MeOH} 345, 300, 225	474[M^+], 250, 224, 167, 149	*Trigynobrathys: H. japonicum*	Ishiguro et al. 1987
22 Sarothralen A	λ^{EtOH} 350, 305, 215	568[M^+]	*Trigynobrathys: H. japonicum*	Ishiguro et al. 1986
23 Sarothralin	λ^{EtOH} 362, 245		*Trigynobrathys: H. japonicum*	Ishiguro et al. 1985
24 Sarothralin G	λ^{EtOH}sh 332, 308	566[M^+], 380, 366, 224, 167, 105, 77	*Trigynobrathys: H. japonicum*	Ishiguro et al. 1990
25 Sarothralen B	λ^{EtOH} 357, 294, 226	566[M^+]	*Trigynobrathys: H. japonicum*	Ishiguro et al. 1986
26 Sarothralen C	λ^{EtOH} 354, 305, 222	584[M^+], 541, 445, 348, 305, 235, 165, 69, 43	*Trigynobrathys: H. japonicum*	Ishiguro et al. 1994
27 Sarothralen D	λ^{EtOH} 354, 303, 225	584[M^+], 541, 460, 348, 305, 235, 165, 69, 43	*Trigynobrathys: H. japonicum*	Ishiguro et al. 1994
28			*Trigynobrathys: H. mutilum*	Schühly et al. 2007
29 [3'mePIB]-[1'pren3'4me4'oxoPIB]		499[M-H]$^-$***, 209, 221, 227, 289	*Brathys: H. gentianoides*	Crispin et al. 2013
30 [3'3'4me6'oxoPIB]-[3'prenPIB]		513[M-H]$^-$***, 237, 249, 263, 275	*Brathys: H. gentianoides*	Crispin et al. 2013
31[1'3'pren45'me4'oxoPIB]-[3'prenPIB]		567[M-H]$^-$ ***,263, 291, 303	*Brathys: H. gentianoides*	Crispin et al. 2013

*Identification by isolation or HPLC detection, **Described by this report, ***ESI-MS in negative mode, Abbreviations: **29** (3,5-dihydroxy-4-isobutyryl-2-methyl-4-(3-methylbut-2-enyl)-6-(2,4,6-trihydroxy-3-isobutyryl-5-methylbenzyl)cyclohexa-2,5-dienone); **30** (3,5-dihydroxy-4,4-dimethyl-2-(2-methylbutanoyl)-6-(2,4,6-trihydroxy-3-isobutyryl-5-(3-methylbut-2-enyl)benzyl)cyclohexa-2,5-dienone); **31** (3,5-dihydroxy-2-methyl-4-(3-methylbut-2-enyl)-4-(2-methylbutanoyl)-6-(2,4,6-trihydroxy-3-isobutyryl-5-(3-methylbut-2-enyl)benzyl)cyclohexa-2,5-dienone

Table 2 ^{1}H NMR data of compounds **1**, **2**, **4** to **11**

H	1[a]	2[a]	4[b]	5[b]	6[b]	7[d]	8[c]	9[d]	10[d]	11[d]
4-Me	1.45 *br s*[e]	1.47 *br s*	1.49 *br s*	1.49 *br s*	1.49 *br s*	1.53 *s*	1.56 *br s*	1.63 *s*	1.52 *s*	1.52 *s*
	1.52 *br s*	1.54 *br s*	1.49 *br s*	1.49 *br s*	1.49 *br s*	-	1.56 *br s*	-	-	-
7	3.30 *s*	3.32 *s*	3.53 *br s*	3.53 *br s*	3.52 *br s*	3.53 *s*	3.54 *br s*	3.59 *s*	3.54 *s*	3.55 *s*
	3.33 *s*	3.35 *s*	3.53 *br s*	3.53 *br s*	3.52 *br s*	3.53 *s*	3.54 *br s*	3.59 *s*	3.54 *s*	3.55 *s*
9	2.70 *s;*	3.20 *q J*=7.2	3.25 *q J*=7.5	3.25 *q J*=7.5	2.74 *s*	2.69 *s*	2.75 *s*	2.69 *s*	2.69 *s*	2.69 *s*
	2.57 *s*									
10	-	1.18 *t*	1.18 *t*	1.18 *t*	-	-	-	-	-	-
		J=7.2	*J*=7.5	*J*=7.5						
2'-Me	-	-	1.49 *br s*	1.49 *br s*	1.49 *br s*	1.50 *s*	1.56 *br s*	1.56 *s*	1.86 *br s*	1.65 *s*
			1.49 *br s*	1.49 *br s*	1.49 *br s*	1.51 *s*	1.56 *br s*	1.57 *s*	1.86 *br s*	1.76 *s*
3'	-	-	5.44 *d*	5.44 *d*	5.44 *d*	5.62 *d*	5.50 *d*	5.64 *d*	5.64 *br t*	5.12 *br t*
			J=9.9	*J*=9.9	*J*=9.9	*J*=9.9	*J*=9.9	*J*=9.9	*J*=7.2	*J*=7.2
4'	-	-	6.69 *d*	6.68 *d*	6.68 *d*	6.66 *d*	6.72 *d*	6.67 *d*	4.81 *d*	3.39 *d*
			J=9.9	*J*=9.9	*J*=9.9	*J*=9.9	*J*=9.9	*J*=9.9	*J*=7.2	*J*=7.2
10'	-	-	-	-	-	-	-	-	6.20 *s*	-
12'	-	-	3.12 *q*	2.69 *s*	2.69 *s*	2.69 *s*	2.75 *s*	2.70 *s*	2.69 *s*	2.69 *s*
			J=7.5							
13'	-	-	1.20 *t*	-	-	-	-	-	-	-
			J=7.5							
1''	-	-	-	-	-	2.69	-	2.69	2.65 *br d*	2.69
						buried[f]		buried[f]	*J*=7.2	buried[f]
2''	-	-	-	-	-	4.65 *br t*	-	4.70 *br t*	4.68 *br t*	4.67 *br t*
						J=8.4		*J*=8.1	*J*=7.2	*J*=7.2
3''-Me	-	-	-	-	-	1.33 *s*	-	1.36 *s*	1.38 *s*	1.34 *s*
	-	-	-	-	-	1.38 *s*	-	1.44 *s*	1.44 *s*	1.39 *s*
3-OH	12.75 *s*	12.27 *s*	9.95 *s*	9.90 *s*	9.94 *s*	9.77 *s*	9.04 *s*	9.10 *s*	9.09 *s*	9.97 *br s*[g]
5-OH	18.40 *s*	18.49 *s*	18.49 *s*	18.49 *s*	18.42 *s*	17.90 *s*	18.47 *s*	18.57 *s*	18.58 *s*	18.39 *s*
5'-OH	-	-	11.44 *s*	11.47 *s*	11.47 *s*	11.38 *s*	-	-	-	11.44 *br s*[g]
7'-OH	-	-	16.01 *s*	15.86 *s*	15.88 *s*	16.04 *s*	11.64 *s*	11.74 *s*	11.61 *s*	15.80 *br s*[g]
9'-OH	-	-	-	-	-	-	14.06 *s*	14.08 *s*	13.68 *s*	6.42 *br s*[g]

[a]NMR data recorded at 400 MHz in $CDCl_3$ (Feng and E-li 2007)
[b]NMR data recorded at 300 MHz in $CDCl_3$ (Jayasuriya et al. 1989)
[c]NMR data recorded at 300 MHz in $CDCl_3$ (Jayasuriya et al. 1994)
[d]NMR data recorded at 300 MHz in Me_2CO-d_6 (Jayasuriya et al. 1991)
[e]Chemical shifts in ppm and coupling constants in Hz
[f]Buried under the signal of δ 2.69
[g]Signals observed in $CDCl_3$
-Absent proton at this position

Table 3 [1]H NMR data of compounds **3**, **12** to **18**

H	3[a]	12[b]	13[c]	14[d]	15[c]	16[b]	17[e†]	18[f]
4-Me	1.48 *s*[g]	1.45 *br s*	-	1.48 *br s*	1.51 *s*	1.50 *s*	1.26 *s*[‡]	1.52 *s*
	1.54 *s*	1.51 *br s*	-	1.48 *br s*	-	1.50 *s*	-	1.52 *s*
6-Me	-	-	1.17 *s*	-	-	-	-	-
7	3.35 *s*	3.53 *br s*	3.41 *s*	3.54 *br s*	3.55 *s*	3.52 *br m*	3.55 *br s*	3.58 *d J*=17
	3.35 *s*	3.53 *br s*	3.41 *s*	3.54 *br s*	3.55 *s*	3.52 *br m*	3.55 *br s*	3.48 *d J*=17
9	4.20 *sep J*=7	4.19 *sep J*=7	3.98 *sep J*=7	4.21 *sep J*=7	4.19 *sep J*=7	4.19 *sep J*=7	4.20 *sep J*=7	4.16 *m*
9-Me	1.18*d J*=7	1.17 *d J*=7[h]	1.05 *d J*=7	1.21 *d J*=7[h]	1.16 *d J*=7	1.17 *d J*=7	1.17 *d J*=7	1.17 *d J*=7[h]
	1.18*d J*=7	1.17 *d J*=7[h]	1.07 *d J*=7	1.21 *d J*=7[h]	1.17 *d J*=7	1.17 *d J*=7	1.17 *d J*=7	1.17 *d J*=7[h]
2'-Me	-	1.79 *s*	1.57 *s*	1.48 *s*	1.53 *s*	1.55 *s*	1.52 unres.	1.31 *s*
	-	1.85 *s*	1.72 *s*	1.48 *s*	1.53 *s*	1.55 *s*	1.52 unres.	1.59 *s*
3'	-	5.22 *pseudo t J*=7	5.22 *br t J*=7	5.44 *d J*=10	5.59 *d J*=10	5.46 *d J*=10	5.48 *d J*=10	1.92 *m*
4'	-	3.45 *d J*=7	3.20 *br d J*=7	6.70 *d J*=10	6.66 *d J*=10	6.71 *d J*=10	6.74 *d J*=10	2.66 *ddd J*=13, 13, 6
12'	-	3.92 *sep J*=7	4.19 *sep J*=7	3.90 *sep J*=7	4.00 *sep J*=7	4.05 *sep J*=7	4.11 *sep J*=7	4.08 *m*
12'-Me	-	1.18 *d J*=7[h]	1.11 *d J*=7	1.18 *d J*=7[h]	1.20 *d J*=7	1.17 *d J*=7	1.18 *d J*=7	1.15 *d J*=7[h]
	-	1.18 *d J*=7[h]	1.11 *d J*=7	1.18 *d J*=7[h]	1.21 *d J*=7	1.17 *d J*=7	1.18 *d J*=7	1.15 *d J*=7[h]
13'	-	-	-	-	-	-	-	1.24 *m*
	-	-	-	-	-	-	-	2.55 *dd J*=13, 6
14'-Me	-	-	-	-	-	-	-	1.07 *s*
	-	-	-	-	-	-	-	1.16 *s*
15'	-	-	-	-	-	-	-	1.22 *m*
	-	-	-	-	-	-	-	1.58 *m*
1''	-	-	2.41 *br d J*=7	-	2.8-2.5 *m*	-	2.8-2.5 *m*	-
2''	-	-	4.81 *br t J*=7	-	4.60 *br t*	-	4.8-4.4 *m*	-
3''-Me	-	-	1.39 *br s*	-	1.31 *s*	-	1.49 unres.	-
	-	-	1.44 *br s*	-	1.34 *s*	-	1.59 unres.	-
3-OH	12.34 *s*	10.10 *br s*	n.o.	9.94 *s*	9.90 *s*	9.05 *s*	9.00 *s*	9.42 *s*
5-OH	18.76 *s*	18.78 *br s*	19.40 *s*	18.68 *s*	18.80 *s*	18.80 *s*	18.64 *s*	18.80 *s*
5'-OH	-	11.50 *br s*	n.o.	12.25 *s*	11.40 *s*	-	-	-
7'-OH	-	16.18 *br s*	14.00 *s*	16.16 *s*	16.35 *s*	11.69 *s*	11.46 *s*	11.46 *s*
9'-OH	-	6.40 *br s*	n.o.	-	-	14.14 *s*	14.13 *s*	14.07 *s*

[a]NMR data recorded at 400 MHz in $CDCl_3$ (Bernardi 2007)
[b]NMR data recorded at 200 MHz in $CDCl_3$ (Rocha et al. 1995)
[c]NMR data recorded at 200 MHz in Me_2CO-d_6 (Rocha et al. 1996)
[d]NMR data recorded at 400 MHz in $CDCl_3$ (Nör 2007)
[e]NMR data recorded at 200 MHz in $CDCl_3$ (Rocha et al. 1996)
[f]NMR data recorded at 500 MHz in Me_2CO-d_6 (Rocha et al. 1995)
[g]Chemical shifts in ppm and coupling constants in Hz
[h]These attributions can be reversed
†Quaternary methyl groups were better resolved in Me_2CO-d_6: δ 1.55 (*s*, 4-Me), 1.46 (*s*, 2'-Me_2), 1.23 and 1.31 (2*s*, 3''-Me_2)
†Hydroxyl resonances in Me_2CO-d_6: δ 18.66, 11.71, 14.02 and 9.15 (br)
‡Tentative assignment
-Absent proton at this position

Table 4 ^{1}H NMR data of compounds **19** to **27**

H	19[a]	20[a]	21[a]	22[b]	23[c]	24[d]	25[b]	26[e]	27[e]
4-Me	1.22 *s*[f]	1.19 *s*	1.20 *s*	1.27 *s*	1.50 *s*	1.50 *s*	1.43 *s*	1.48 *br s*	1.49 *br s*
	1.22 *s*	1.19 *s*	1.20 *s*	1.27 *s*	1.56 *s*	1.50 *s*	1.43 *s*	1.55 *br s*	1.49 *br s*
7	3.46 *s*	3.46 *s*	3.47 *s*	3.54 *s*	3.58 *s*	3.57 *s*	3.53 *s*	3.57 *s*	3.52 *s*
	3.46 *s*	3.46 *s*	3.47 *s*	3.54 *s*	3.60 *s*	3.57 *s*	3.53 *s*	3.57 *s*	3.52 *s*
9	3.91 *sep*	3.94 *sep*	3.82 *sex*	4.20 *sep*	4.18 *m*	4.21 *m*	3.90 *m*	4.21 *sep*	4.15 *sep*
	J=7.3	*J*=6.6	*J*=7	*J*=7	*J*=6.5			*J*=6.7	*J*=6.7
9-Me	1.09 *d*	1.08 *d*	1.08 *d*	1.15 *d*	1.18 *d*	1.14 *d*	1.17 *d*	1.18 *d*	1.19 *d*
	J=7.3	*J*=6.6	*J*=7	*J*=7	*J*=6.9	*J*=6.3	*J*=6.4	*J*=6.7	*J*=6.7
	1.09 *d*	1.08 *d*	-	1.15 *d*	1.18 *d*	1.14 *d*	1.17 *d*	1.18 *d*	1.19 *d*
	J=7.3	*J*=6.6		*J*=7	*J*=6.9	*J*=6.3	*J*=6.4	*J*=6.7	*J*=6.7
10	-	-	1.47 *m*	-	-	-	-	-	-
	-	-	1.76 *m*	-	-	-	-	-	-
11	-	-	0.86 *t J*=7.3	-	-	-	-	-	-
1'	-	-	-	2.05 *m*	-	2.04 *br s*	1.82 *m*	1.75 *m*	1.78 *m*
2'-Me	-	-	-	1.77 *s*	1.54 *s*	1.72 *s*	1.66 *s*	1.52 *s*	1.53 *s*
	-	-	-	-	1.54 *s*	-	-	-	-
3'	-	-	-	5.25 *m*	4.64 *tq*	5.12 *t*	5.42 *d*	3.94 *br t*	3.98 *br d*
					J=6.1, 1.5	*J*=6.3	*J*=10	unres.	*J*=4.9
4'	-	-	-	3.29 *d*	4.16 *d*	3.36 *d*	6.76 *d*	2.65 *dd*	2.70 *br d*
				J=6.8	*J*=6.1	*J*=6.3	*J*=10	*J*=6.4,17.1	*J*=16.8
	-	-	-	-	-	-	-	2.94 *dd*	2.93 *dd*
								J=5.5, 17.1	*J*=4.9, 16.8
10'	-	-	-	-	6.02 *s*	-	-	-	-
10'-Me	1.95 *s*	1.94 *s*	1.94 *s*	-	-	-	-	-	-
12'	4.16 *sep*	4.07 *sex*	4.06 *sex*	3.95 *sep*	-	-	4.19 *m*	3.91 *sep*	4.12 *m*
	J=6.6	*J*=7.3	*J*=6.7	*J*=7				*J*=6.7	unres.
12'-Me	1.13 *d J*=6.6	1.11 *d J*=7.3	1.12 *d J*=6.7	1.18 *d J*=7	-	-	1.20 *d J*=6.4	1.19 *d J*=6.7	1.19 *d J*=6.7
	1.13 *d J*=6.6	-	-	1.18 *d J*=7	-	-	1.20 *d J*=6.4	1.19 *d J*=6.7	1.19 *d J*=6.7
13'	-	1.38 *m*	1.47 *m*	-	7.35 *m*	7.64 *m*	-	-	-
	-	1.81 *m*	1.76 *m*	-	-		-	-	-
14'	-	0.88 *t J*=7.4	0.88 *t J*=7.3	-	7.46 *m*	7.50 *m*	-	-	-
15'	-	-	-	-	7.46 *m*	7.50 *m*	-	-	-
16'	-	-	-	-	7.46 *m*	7.50 *m*	-	-	-
17'	-	-	-	-	7.35 *m*	7.64 *m*	-	-	-
1''	-	-	-	1.95 *m*	-	1.98 *br s*	2.09 *m*	2.13 *m*	2.16 *m*
2''	-	-	-	5.05 *m*	-	4.96 *m*	5.09 *m*	5.08 *t*	5.09 *dd*
								J=6.7	*J*=7.4, 5.4
3''-Me	-	-	-	1.58 *s*	-	1.52 *s*	1.57 *s*	1.59 *s*	1.60 *s*
	-	-	-	1.65 *s*	-	1.52 *s*	1.57 *s*	1.67 *s*	1.67 *s*
3-OH	n.o.	n.o.	n.o.	10.14 *s*	9.70 *s*	10.11 *s*	10.01 *s*	10.08 *s*	9.03 *s*
5-OH	19.39 *s*	19.39 *s*	19.26 *s*	18.72 *s*	18.75 *s*	18.75 *s*	18.75 *s*	18.70 *s*	18.68 *s*
5'-OH	n.o.	n.o.	n.o.	10.74 *s*	11.43 *s*	11.47 *s*	11.49 *s*	11.47 *s*	-
7'-OH	14.03 *s*	14.03 *s*	13.93 *s*	16.12 *s*	14.65 *s*	16.18 *s*	16.44 *s*	16.32 *s*	11.33 *s*
9'-OH	n.o.	n.o.	n.o.	6.41 *s*	-	11.47 *s*	-	-	14.15 *s*

[a]NMR data recorded at 200 MHz in Me2CO-d6 (Ishiguro et al. 1987)
[b]NMR data recorded at 200 MHz in CDCl3+CD3OD (Ishiguro et al. 1986)
[c]NMR data recorded at 360 MHz in CDCl3 (Ishiguro et al. 1985)
[d]NMR data recorded at 200 MHz in CDCl3 (Ishiguro et al. 1990)
[e]NMR data recorded at 500 MHz in CDCl3 (Ishiguro et al. 1994)
[f]Chemical shifts in ppm and coupling constants in Hz
n.o. Signal not observed
-Absent proton at this position

Table 5 ^{13}C NMR data from compounds **1** to **14**

C	1[a]	2[a]	3[b]	4[c]	5[c]	6[d]	7[e]	8[d]	9[f]	10[f]	11[f]	12[g†]	13[h]	14[i]
1	187.6[j]	187.6	199.8	198.0	198.0	198.6	198.0	198.4	198.0	198.0	199.1	198.9	195.5	199.4
2	110.6	110.8	107.1	108.0	108.0	108.2	111.0	108.2	110.2	110.9	111.0	106.5[x]	109.0[x]	106.8[x]
3	173.5	173.3	173.3	171.6	171.6	171.6	171.0	171.0	170.0	170.5	170.6	182.0	183.8	171.7
4	44.5	44.3	44.5	44.3	44.2	44.1	49.6	44.0	48.8	49.6	49.8	51.9[‡]	57.5	44.3
4-Me	24.2	24.2	24.3	24.8	24.8	25.0	23.3	25.0	23.3	23.4	23.3	24.6	-	24.3
	25.3	25.3	25.4	24.8	24.8	25.0	-	25.0	-	-	-	24.6	-	25.4
5	199.4	198.7	187.4	187.3	187.3	187.2	189.9	187.3	187.9	188.2	188.6	198.9	199.6	187.3
6	108.4	107.8	110.8	111.2	111.2	110.9	114.0	111.0	113.1	113.9	114.4	107.5[x]	105.0[x]	111.2
6-Me	-	-	-	-	-	-	-	-	-	-	-	-	23.8	-
7	18.0	18.0	18.2	16.8	16.8	16.5	16.8	16.8	16.7	17.2	17.4	18.0	18.9	16.9
8	203.1	207.1	210.6	207.1	207.1	203.2	203.3	204.7	203.0	203.7	203.7	209.9	197.3	211.0
9	29.2	34.8; 34.7	36.6	34.8	34.8	29.2	29.1	29.3	29.3	buried*	29.3	32.2	33.5	36.6
9-Me	-	-	18.7	-	-	-	-	-	-	-	-	19.2	19.8[§]	18.9[y]
	-		19.3	-	-	-	-	-	-	-	-	19.2	19.8[§]	18.9[y]
10	-	8.5; 8.2	-	8.6	8.6	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1'	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2'	-	-	-	78.2	78.2	77.9	79.1	80.5	80.6	142.5	132.7	129.1	129.6	78.1
2'-Me	-	-	-	28.1	28.2	27.9	28.0	27.5	27.8	18.2	18.0	17.5	17.8	27.7
	-	-	-	28.1	28.2	27.9	28.0	27.5	27.8	25.9	25.9	25.3	25.9	27.9
3'	-	-	-	124.6	124.7	124.6	126.4	124.0	124.2	118.1[x]	123.9	123.6	125.2	124.6
4'	-	-	-	117.3	117.2	117.0	117.5	117.0	117.0	67.1	22.3	21.4	22.6	117.3
5'	-	-	-	159.1	159.4	159.2	159.8	154.8	154.8	166.1[y]	159.9[x]	157.4[y]	159.0[y]	159.3[z]
6'	-	-	-	106.1	106.0	105.7	106.7	104.6	104.9	105.9[z]	106.9[y]	103.7[x]	107.5[x]	107.1[x]
7'	-	-	-	161.4	161.2	161.0	161.6	160.6	160.8	160.8[y]	161.5[x]	160.8[y]	162.5[y]	162.1[z]
8'	-	-	-	104.4	104.8	104.6	105.4	106.6	106.7	107.3[z]	106.5[y]	102.7[x]	104.9[x]	103.7[x]
9'	-	-	-	155.7	155.9	155.7	156.7	159.8	160.0	161.9[y]	160.0[x]	161.3[y]	163.1[y]	155.4[z]
10'	-	-	-	103.6	103.6	103.4	104.1	101.7	101.8	93.4	108.2[y]	104.6[x]	107.7[x]	103.5[x]
10'-Me	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11'	-	-	-	206.9	203.5	203.2	204.9	203.4	204.9	204.9	205.1	209.9	211.1	210.8
12'	-	-	-	37.1	32.6	32.5	33.0	33.4	33.5	33.2	32.8	38.0	39.2	39.0
12'-Me	-	-	-	-	-	-	-	-	-	-	-	19.2	19.8	19.2[y]
	-	-	-	-	-	-	-	-	-	-	-	19.2	19.8	19.2[y]
13'	-	-	-	8.9	-	-	-	-	-	-	-	-	-	-
14'	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14'-Me	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15'	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16'	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17'	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1''	-	-	-	-	-	-	38.9	-	38.7	38.6	38.8	-	39.5	-
2''	-	-	-	-	-	-	118.2	-	117.0	118.2[x]	118.2	-	121.2	-
3''	-	-	-	-	-	-	136.5	-	136.0	136.4	136.6	-	133.0	-
3''-Me	-	-	-	-	-	-	17.6	-	17.6	17.6	17.6	-	17.8	-
	-	-	-	-	-	-	25.6	-	25.6	25.5	25.6	-	25.9	-

[a]NMR data recorded at 100 MHz in $CDCl_3$ (Feng and E-li 2007)
[b]NMR data recorded at 50 MHz in $CDCl_3$ (Rocha et al. 1995)
[c]NMR data recorded at 75 MHz in $CDCl_3$ (Jayasuriya et al. 1989)
[d]NMR data recorded at 75 MHz in $CDCl_3$ (Jayasuriya et al. 1994)
[e]NMR data recorded at 75 MHz in Me_2CO-d_6 (Jayasuriya et al. 1991)
[f]NMR data recorded at 75 MHz in $CDCl_3$ (Jayasuriya et al. 1991)
[g]NMR data recorded at 50 MHz in DMSO-d_6 (Rocha et al. 1995)
[h]NMR data recorded at 50 MHz in Me_2CO-d_6 (Rocha et al. 1996)
[i]NMR data recorded at 100 MHz in $CDCl_3$ (Nör 2007)
[j]Chemical shifts in ppm
[†]Only two signals observed in the region of δ 190-210. It is assumed that the signal at δ 198.9 can be attributed to C-1 and C-5, while the signal at δ 209.9 can be attributed to C-8 and C-11'. All four resonances were observed when the spectrum was recorded in $CDCl_3$ at δ 211.3, 210.8, 199.3 and 171.6
[‡]Tentative assignment (low intensity); signal observed at δ 44.2 in $CDCl_3$
[§]Broad signal, unresolved
*Buried under acetone signal
[x,y,z]Values with the same superscripts in each column are interchangeable
-Absent carbon at this position

Table 6 ^{13}C NMR data of compounds **15** to **27**

C	15[a]	16[b]	17[a]	18[c]	19[d]	20[d]	21[d]	22[e]	23[f]	24[g]	25[e]	26[h]	27[h]
1	199.2[i]	199.1	199.1	200.1	197.4	197.5	200.6	199.4	199.6	199.5	199.4	199.5	199.8
2	109.8[x]	106.9[x]	109.8[x]	107.9	105.9	105.9	105.8	106.3	106.2	107.8	106.1	106.1	105.9
3	170.7	170.9	170.2	172.0	183.6	183.7	183.9	171.7	171.5	171.2	171.1	171.8	171.1
4	49.8	44.1	49.7	44.8	53.7	53.6	53.6	44.4	44.4	44.5	44.4	44.4	44.1
4-Me	22.8	24.5	22.9	24.9	25.3	25.3	25.6	25.6	25.5*	25.8	25.6	24.4	25.7
	-	25.0	-	25.1	25.3	25.3	25.6	25.6	25.5*	25.8	25.6	25.6	25.7
5	188.5	187.2	188.7	188.2	196.7	196.6	196.5	187.4	187.3	187.2	187.3	187.4	187.4
6	114.2	111.3	114.1	112.2	109.1	109.1	109.1	107.2	111.2	111.2	111.3	111.3	111.7
6-Me	-	-	-	-	-	-	-	-	-	-	-	-	-
7	16.9	17.0	17.1	18.0	19.1	19.1	19.1	18.8	16.8	16.7	16.9	17.0	17.1
8	211.9[y]	211.9	212.3[y]	211.6	211.3	211.0	208.3	211.4	210.8	211.2	210.9	210.8	212.0
9	37.2	36.8	37.4	37.4	33.4	33.4	40.3	36.3	36.7	36.3	36.6	36.6	39.5
9-Me	19.4	18.9[y]	19.5	19.6	19.9	19.9	17.3	19.1	18.0*	19.5	17.5	19.2	19.0
	19.5	18.9[y]	19.5	19.7	19.9	19.9	-	19.5	19.2*	19.6	19.1	19.2	19.3
10	-	-	-	-	-	-	27.8	-	-	-	-	-	-
11	-	-	-	-	-	-	12.2	-	-	-	-	-	-
1'	-	-	-	-	-	-	-	22.2	-	27.0[x]	41.8	37.6	36.5
2'	79.2	80.5	81.5	84.3	-	-	-	132.2	137.2	133.8	81.0	80.7	83.2
2'-Me	27.8	27.7	27.6	19.9	-	-	-	19.6	19.0	18.0	26.8	25.6	24.4
	27.9	27.7	28.0	28.2	-	-	-	-	19.0	-	-	-	-
3'	126.1	124.1	125.8	51.6	-	-	-	122.2	118.3	125.3	123.1	66.7	67.7
4'	117.5	117.1	117.2	36.5	-	-	-	38.7	65.1	39.8	123.7	26.2	26.2
5'	160.0[z]	154.6[z]	160.9[z]	157.2	158.9[x]	158.9[x]	159.6[x]	161.7[x]	164.9[x]	161.9[y]	162.2	162.1	154.4
6'	107.2[x]	104.9[x]	106.3[x]	105.6[x]	103.7[y]	103.9[y]	103.9[y]	105.7	104.5	103.7	103.3[x]	103.7	107.1
7'	162.7[z]	160.1[z]	161.0[z]	158.7	163.4[x]	163.3[x]	162.7[x]	160.7[x]	161.0[x]	159.2[y]	159.4	162.0	158.2
8'	104.3[x]	105.7[x]	106.2[x]	105.8[x]	104.0[y]	103.6[y]	104.5[y]	108.3	107.1	106.3	108.2	107.2	104.5
9'	156.1[z]	160.4[z]	155.9[z]	164.3	162.9[x]	162.8[x]	162.8[x]	158.1[x]	162.0[x]	159.2[y]	155.7	154.6	163.3
10'	104.2[x]	102.1[x]	102.8[x]	106.4	105.2[y]	105.9[y]	105.3[y]	111.5	94.2	106.3	103.6[x]	100.1	98.8
10'-Me	-	-	-	-	8.1	8.1	8.1	-	-	-	-	-	-
11'	210.8[y]	210.9	211.0[y]	212.2	208.6	208.9	208.3	210.8	199.4	202.5	210.7	210.8	212.0
12'	39.7	39.5	40.1	40.0	39.3	46.0	46.1	37.1	142.0	140.9	38.9	39.1	39.5
12'-Me	18.9	19.2	18.7	19.1	19.9	17.4	17.3	19.3	-	-	19.3	19.8	19.4
	19.8	19.2	19.6	19.1	19.9	-	-	19.3	-	-	19.3	19.8	19.4
13'	-	-	-	47.3	-	28.0	27.8	-	127.3	128.0	-	-	-
14'	-	-	-	37.8	-	12.2	12.2	-	127.5	128.5	-	-	-
14'-Me	-	-	-	32.2	-	-	-	-	-	-	-	-	-
	-	-	-	32.5	-	-	-	-	-	-	-	-	-
15'	-	-	-	41.9	-	-	-	-	130.2	132.0	-	-	-
16'	-	-	-	-	-	-	-	-	127.5	128.5	-	-	-
17'	-	-	-	-	-	-	-	-	127.3	128.0	-	-	-
1''	39.3	-	39.1	-	-	-	-	26.4	-	29.8[x]	23.2	22.1	22.1
2''	118.2	-	118.1	-	-	-	-	121.1	-	121.1	118.0	123.6	123.2
3''	136.5	-	136.7	-	-	-	-	140.7	-	139.0	132.0	132.4	132.8
3''-Me	17.5	-	17.6	-	-	-	-	19.6	-	22.0	19.6	17.6	17.7
	25.6	-	25.6	-	-	-	-	19.6	-	22.0	19.6	25.7	25.7

[a]NMR data recorded at 50 MHz in Me_2CO-d_6 (Rocha et al. 1996)
[b]NMR data recorded at 50 MHz in $CDCl_3$ (Rocha et al. 1995)
[c]NMR data recorded at 50 MHz in Me_2CO-d_6 (Rocha et al. 1995)
[d]NMR data recorded at 25.2 MHz in Me_2CO-d_6 (Ishiguro et al. 1987)
[e]NMR data recorded at 25.2 MHz in $CDCl_3$ (Ishiguro et al. 1986)
[f]NMR data recorded at 25.2 MHz in $CDCl_3$ (Ishiguro et al. 1985)
[g]NMR data recorded at 25.2 MHz in $CDCl_3$ (Ishiguro et al. 1990)
[h]NMR data recorded at 125 MHz in $CDCl_3$ (Ishiguro et al. 1994)
[i]Chemical shifts in ppm
[x,y,z]Values with the same superscripts in each column are interchangeable
*Original reference assigns δ 25.5 for 9-Me and δ 18.0; 19.2 for 4-Me
-Absent carbon at this positi

Como parte do presente capítulo são apresentados espectros de ressonância magnética nuclear (RMN) 1D (^{1}H, ^{13}C, DEPT) e 2D (COSY, HETCOR, HMBC) de cinco derivados de acilfloroglucinol diméricos, previamente descrevidos para *H. uliginosum* (PARKER e JOHNSON, 1968) e *H. brasiliense* (ROCHA *et al*. 1995; ROCHA *et al*. 1996), uliginosina A, uliginosina B, isouliginosina B, hiperbrasilol B e isohiperbrasilol B, mas relatados pela primeira vez em quatro espécies Peruanas de *Hypericum*.

	Uliginosin A	**Uliginosin B**	**Isouliginosin B**	**Hyperbrasilol B**	**Isohyperbrasilol B**
H. andinum	X	X	X		
H. brevistylum	X	X	X		
H. laricifolium		X	X	X	X
H. silenoides	X	X	X		

Estes espectros foram obtidos num espectrômetro FTNMR Anasazi de 60 MHz (Frequência de trabalho nos experimentos de ^{1}H, e ^{13}C: 60 e 15 MHz respectivamente. Experimentos realizados na Central Analítica II da Faculdade de Farmácia - UFRGS) e num espectrômetro Varian MR400 de 400 MHz (Frequência de trabalho nos experimentos de ^{1}H, e ^{13}C: 399.736 e 100.523 MHz respectivamente. Experimentos realizados no Laboratório Regional de Nanotecnologia – LRNANO - UFRGS). Este material é sumarizado no seguiste ordenamento:

Uliginosina A: Figura 1.1 - 1.11 [$CDCl_3$ e acetona-d_6]

Uliginosina B: Figura 2.1 - 2.18 [$CDCl_3$, acetona-d_6, CD_3OD, C_5D_5N e CD_3SOCD_3]

Isouliginosina B: Figura 3.1 - 3.10 [$CDCl_3$ e acetona-d_6]

Hiperbrasilol B: Figura 4.1 - 4.10 [$CDCl_3$ e acetona-d_6]

Isohiperbrasilol B: Figura 5.1 - 5.10 [$CDCl_3$ e acetona-d_6]

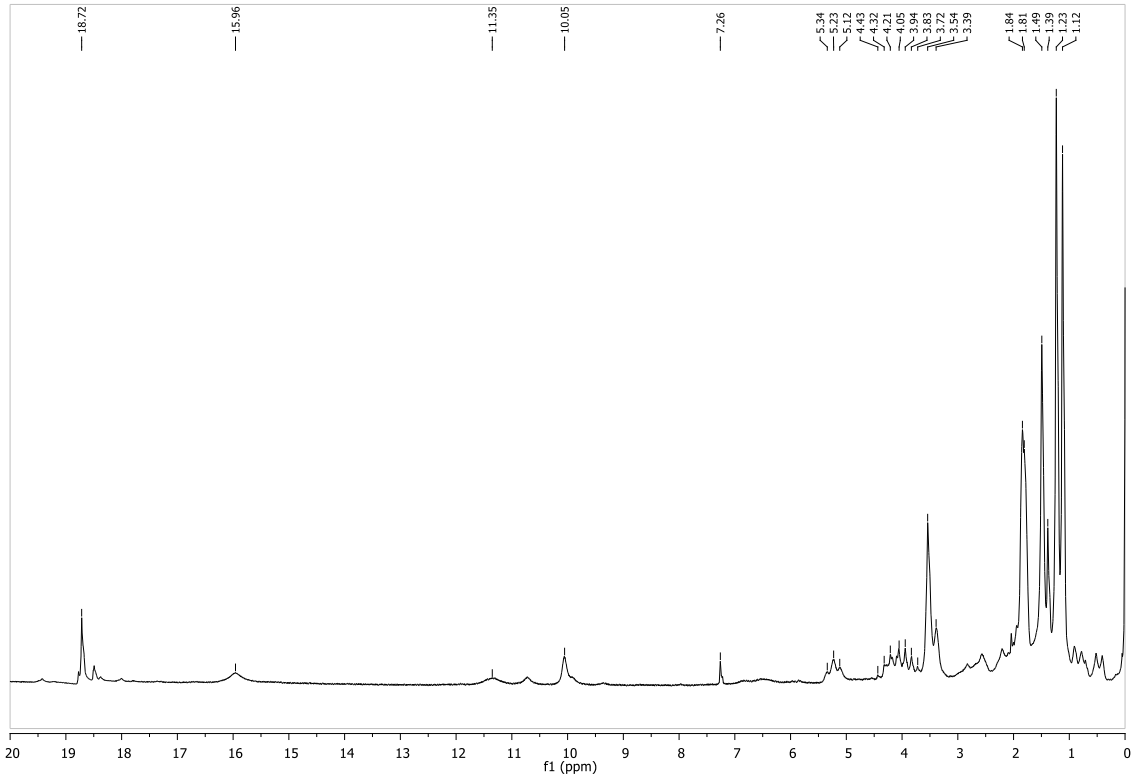

Figura 1.1 Espectro de RMN-^{1}H de **uliginosina A** em $CDCl_3$ a 60 MHz

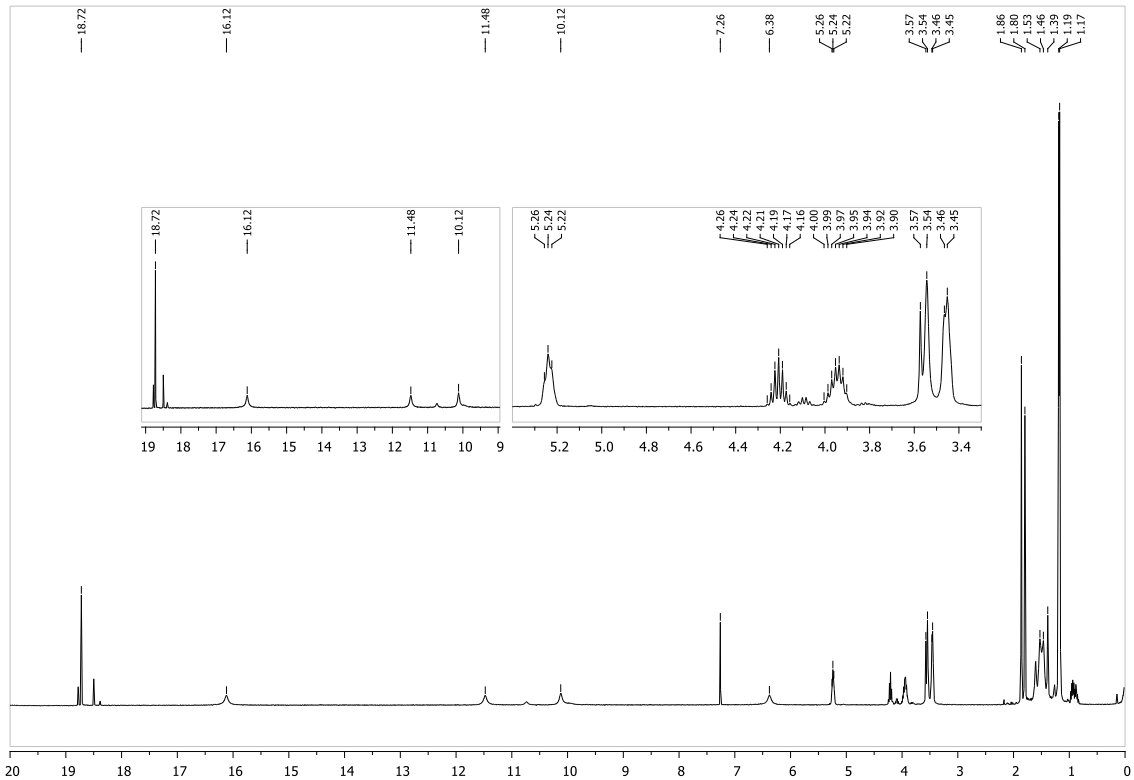

Figura 1.2 Espectro de RMN-^{1}H de **uliginosina A** em $CDCl_3$ a 400 MHz

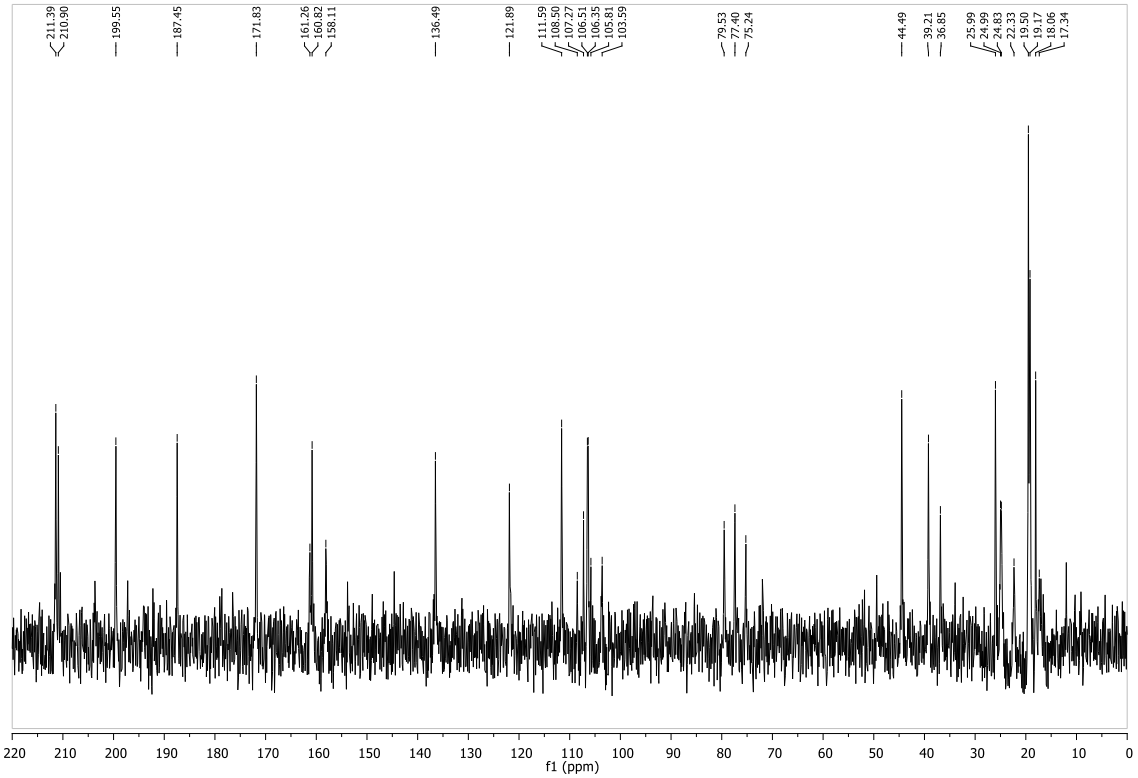

Figura 1.3 Espectro de RMN-^{13}C de **uliginosina A** em $CDCl_3$ a 15 MHz

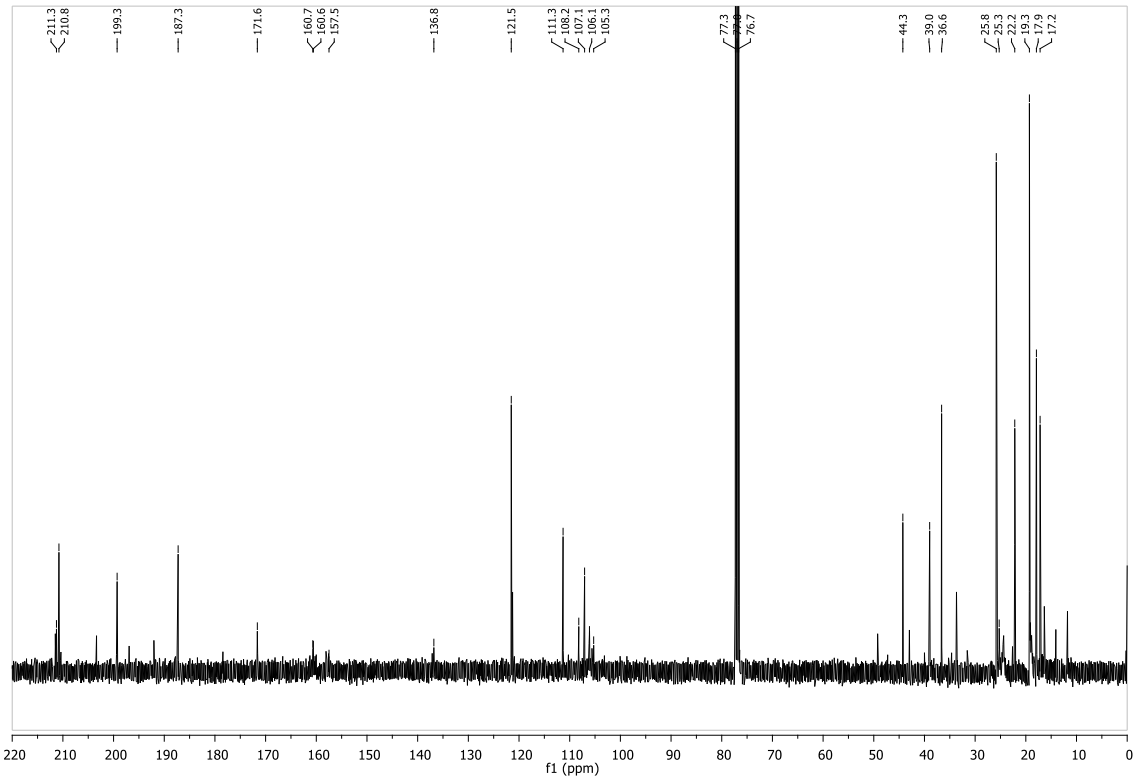

Figura 1.4 Espectro de RMN-^{13}C de **uliginosina A** em $CDCl_3$ a 100 MHz

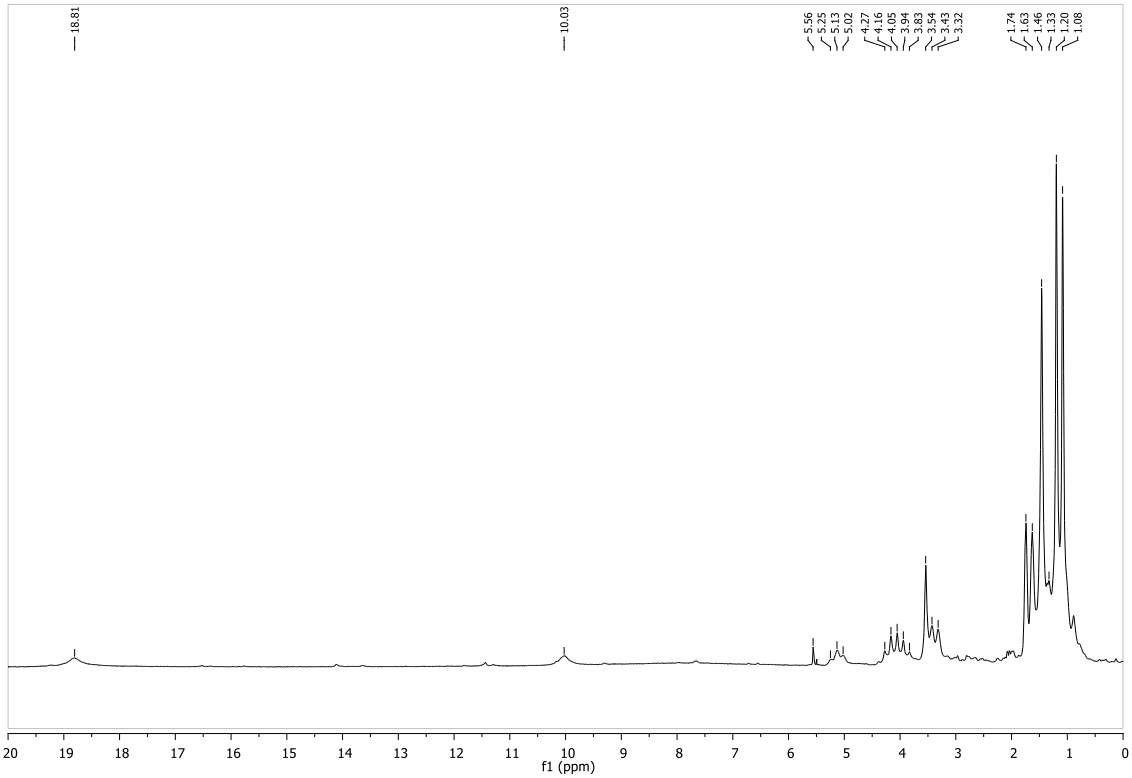

Figura 1.5 Espectro de RMN-^{1}H de **uliginosina A** em acetona-d_6 a 60 MHz

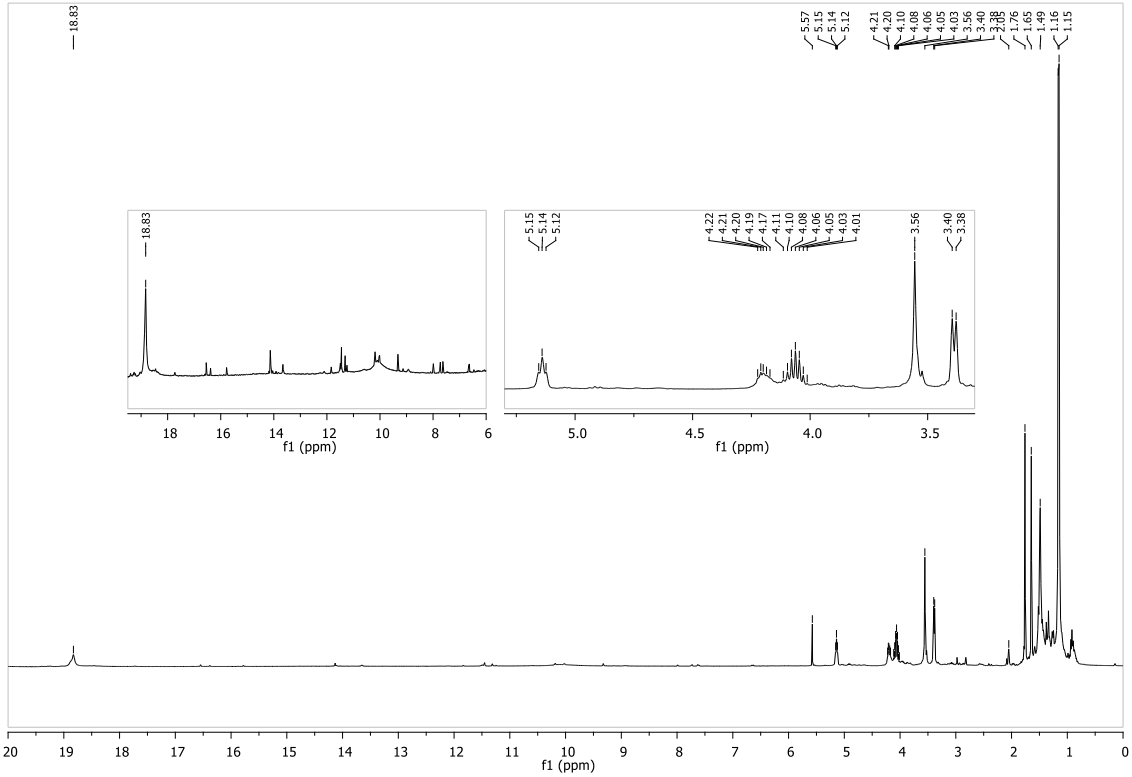

Figura 1.6 Espectro de RMN-^{1}H de **uliginosina A** em acetona-d_6 a 400 MHz

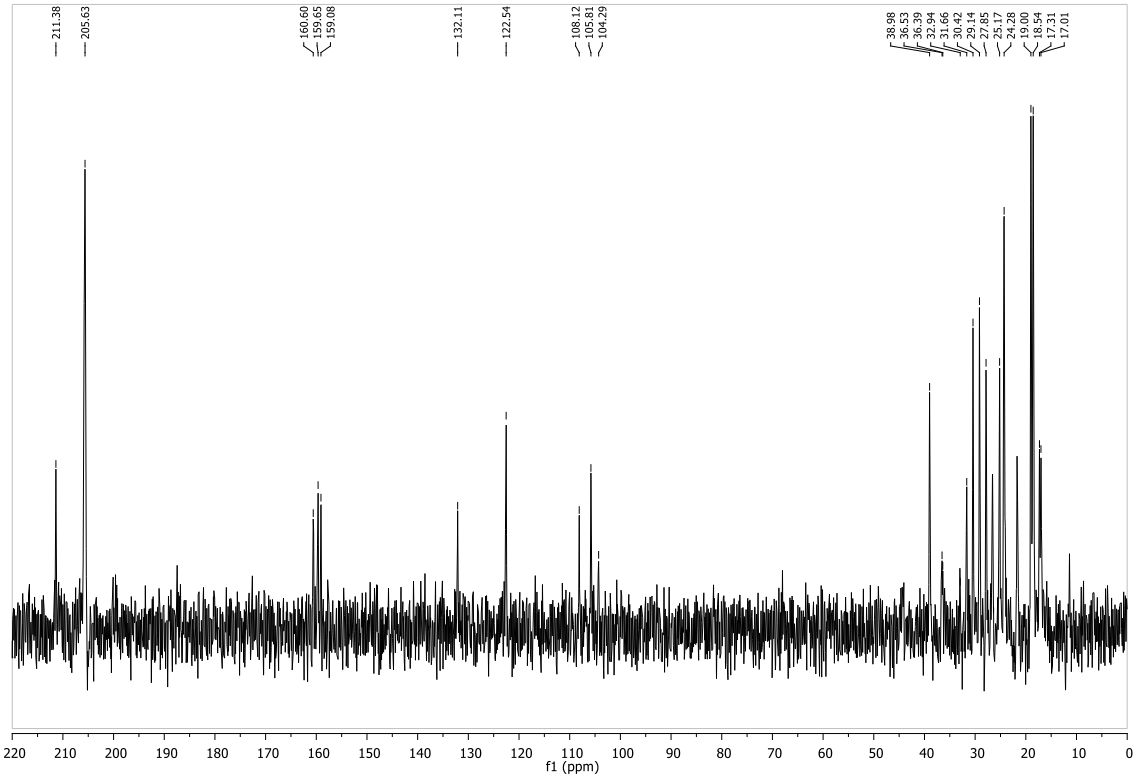

Figura 1.7 Espectro de RMN-^{13}C de **uliginosina A** em acetona-d_6 a 15 MHz

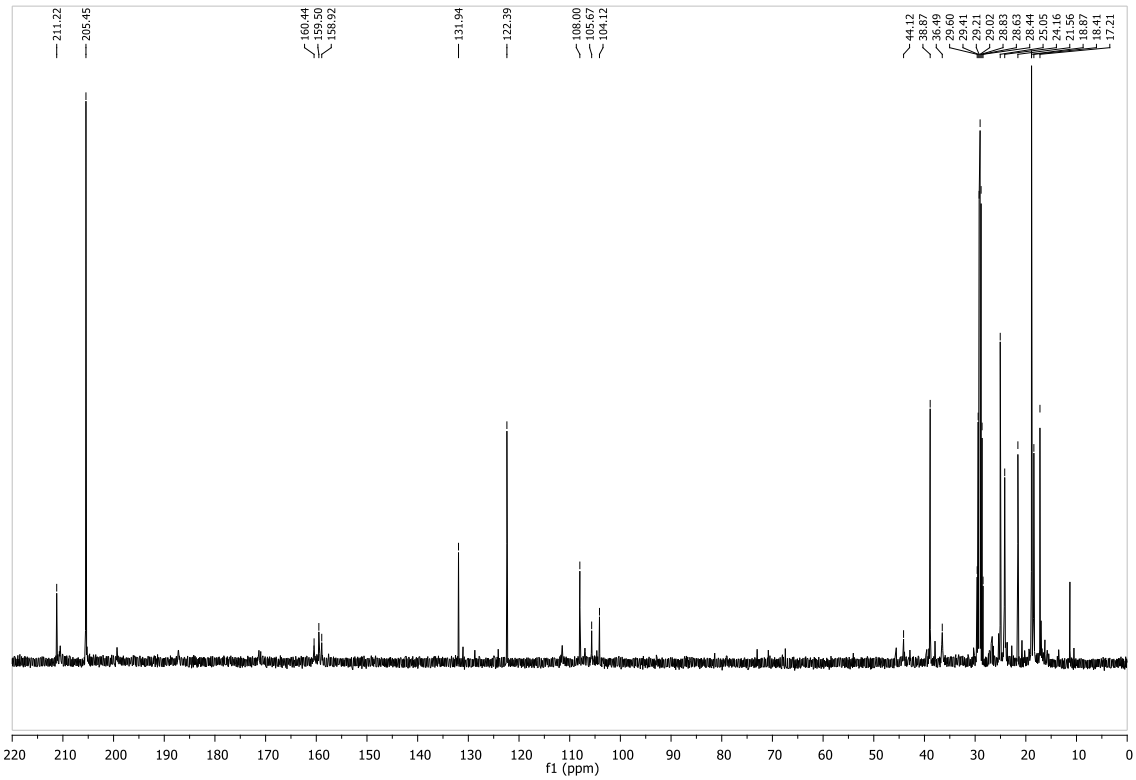

Figura 1.8 Espectro de RMN-^{13}C de **uliginosina A** em acetona-d_6 a 100 MHz

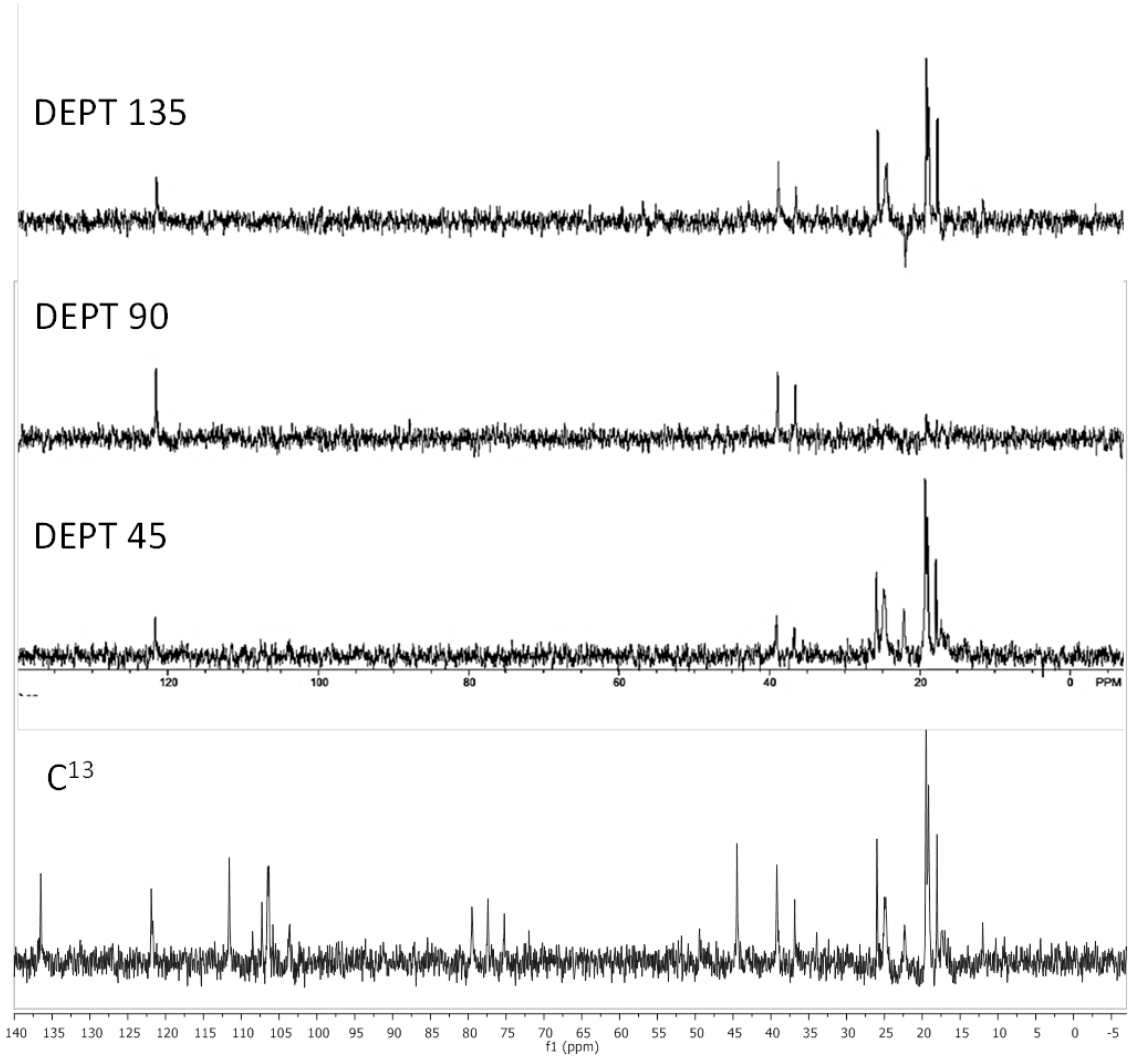

Figura 1.9 Espectro de RMN-^{13}C-DEPT de **uliginosina A** em $CDCl_3$ a 15 MHz

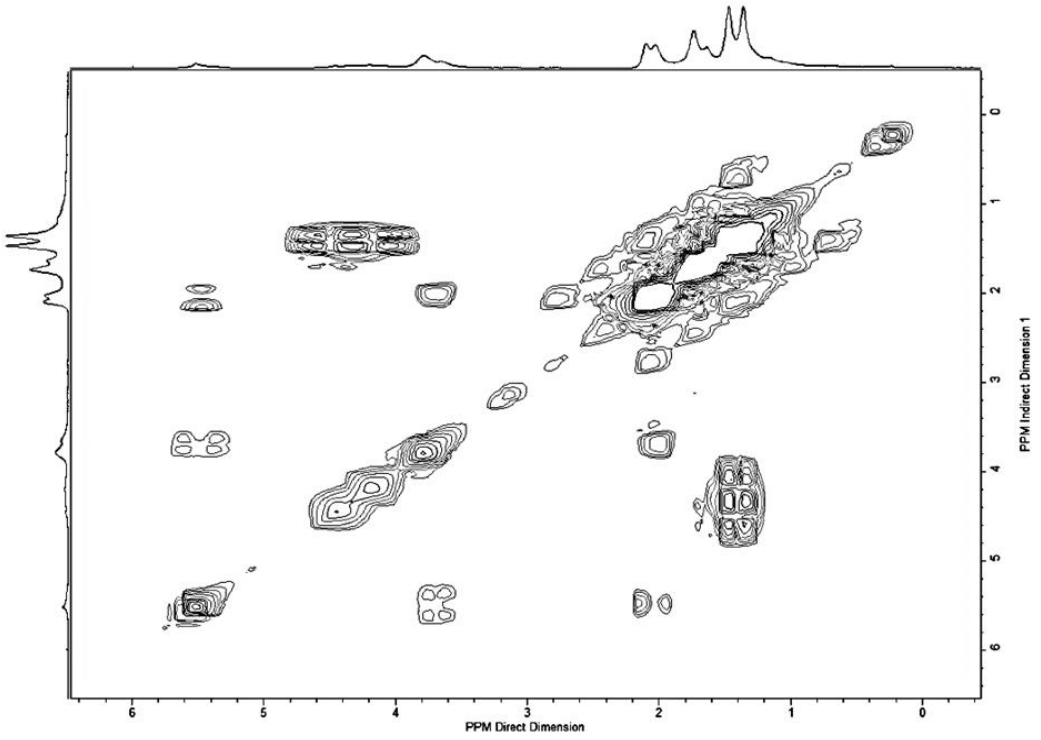

Figura 1.10 Espectro de correlação H-H COSY de **uliginosina A** em $CDCl_3$

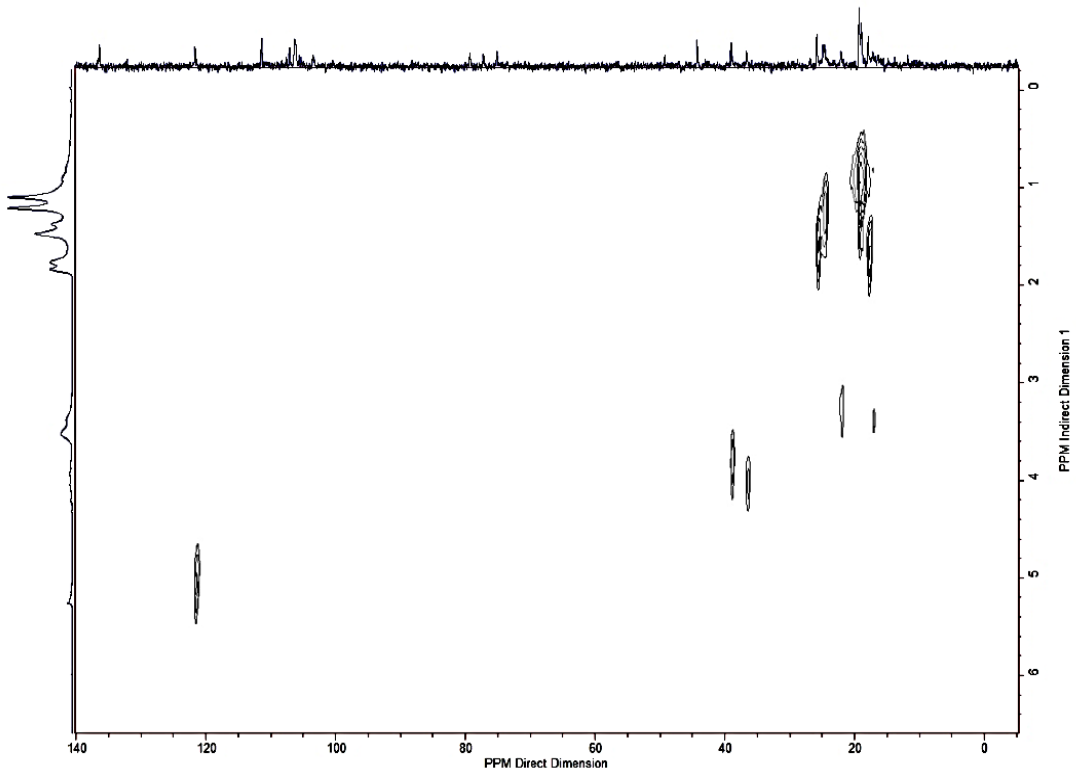

Figura 1.11 Espectro de correlação H-C HETCOR de **uliginosina A** em $CDCl_3$

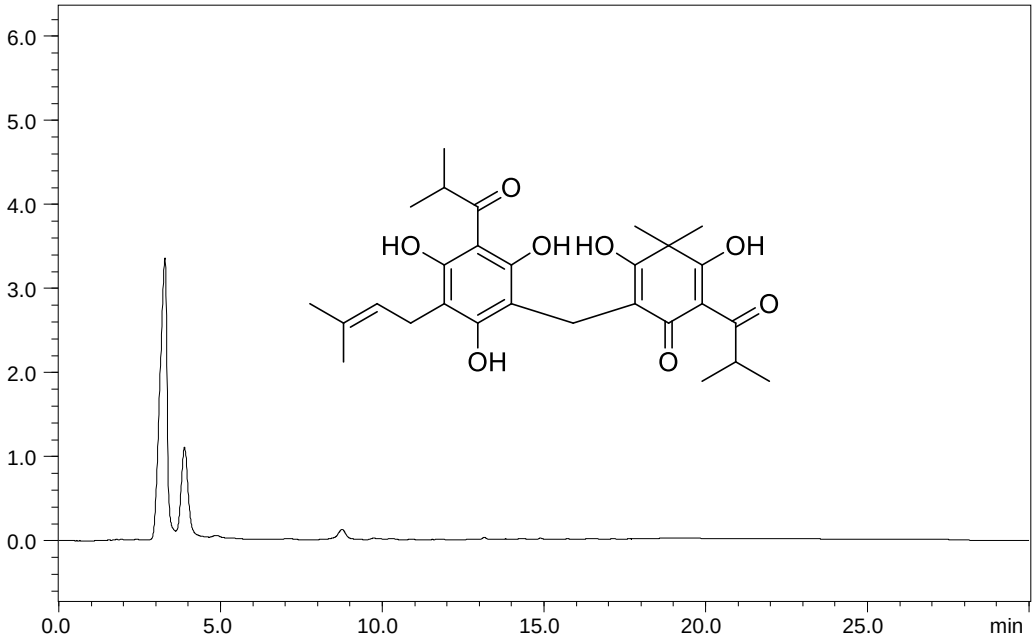

Figure 1.12 Cromatograma por HLPC de uliginosina A

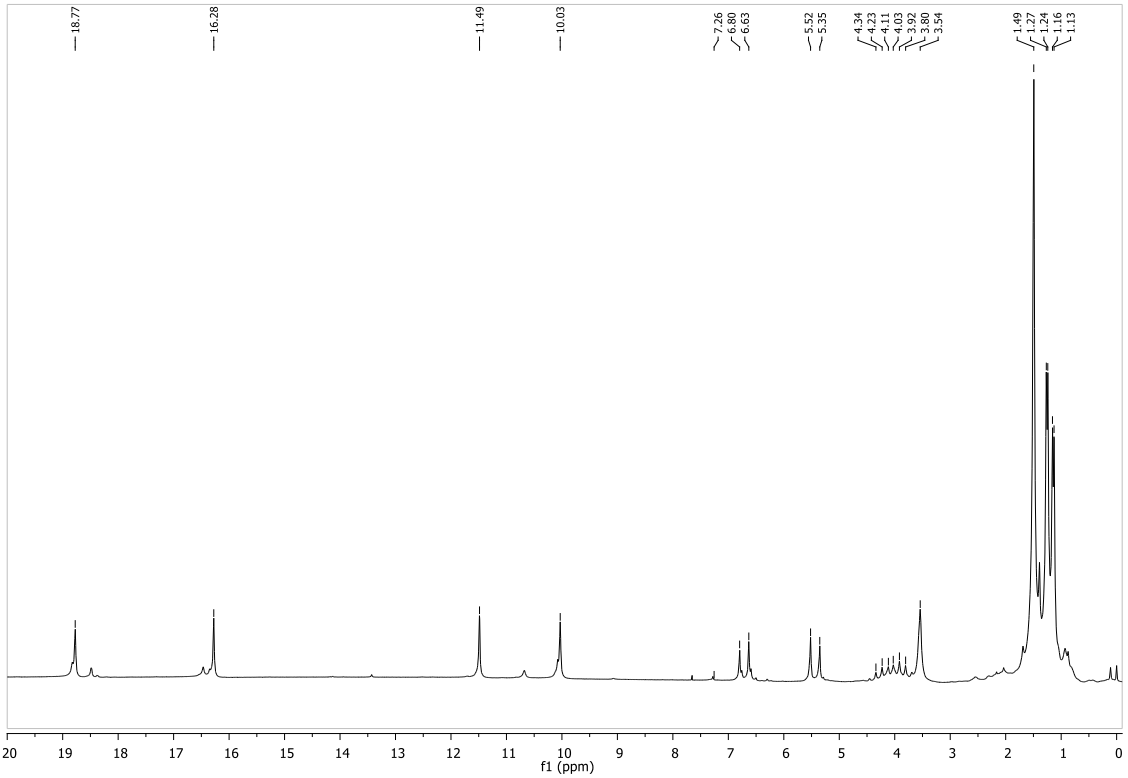

Figura 2.1 Espectro de RMN-^{1}H de **uliginosina B** em $CDCl_3$ a 60 MHz

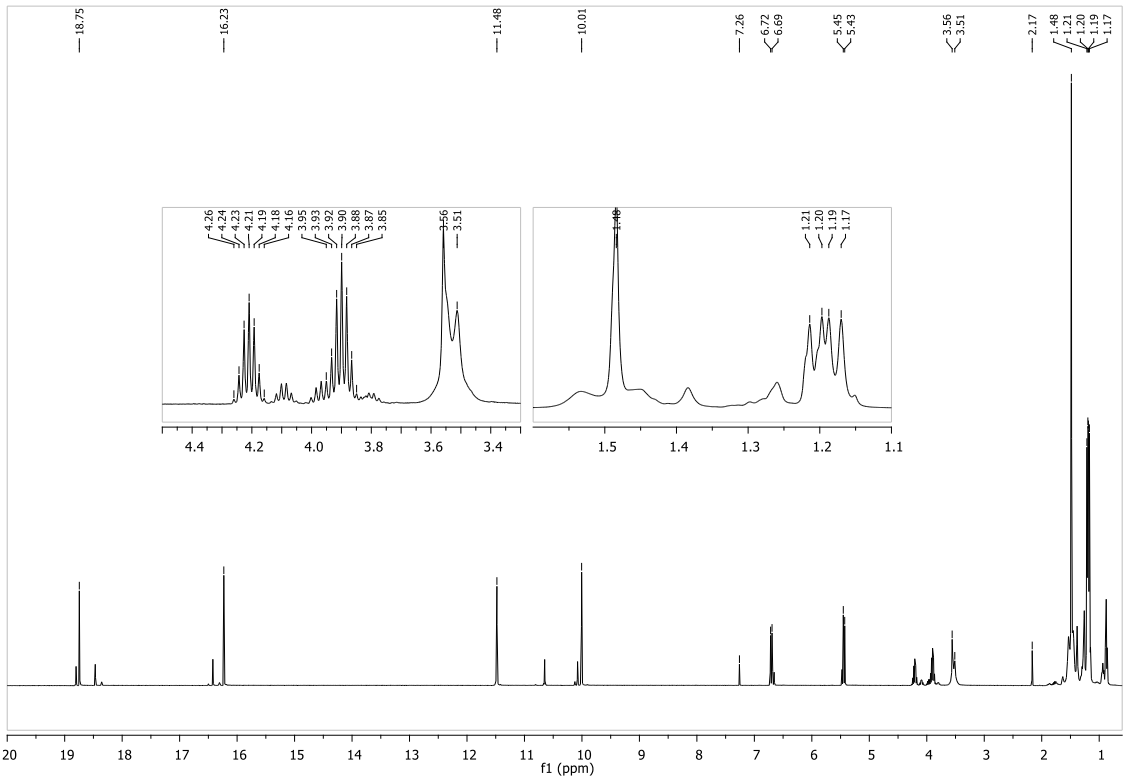

Figura 2.2 Espectro de RMN-^{1}H de **uliginosina B** em $CDCl_3$ a 400 MHz

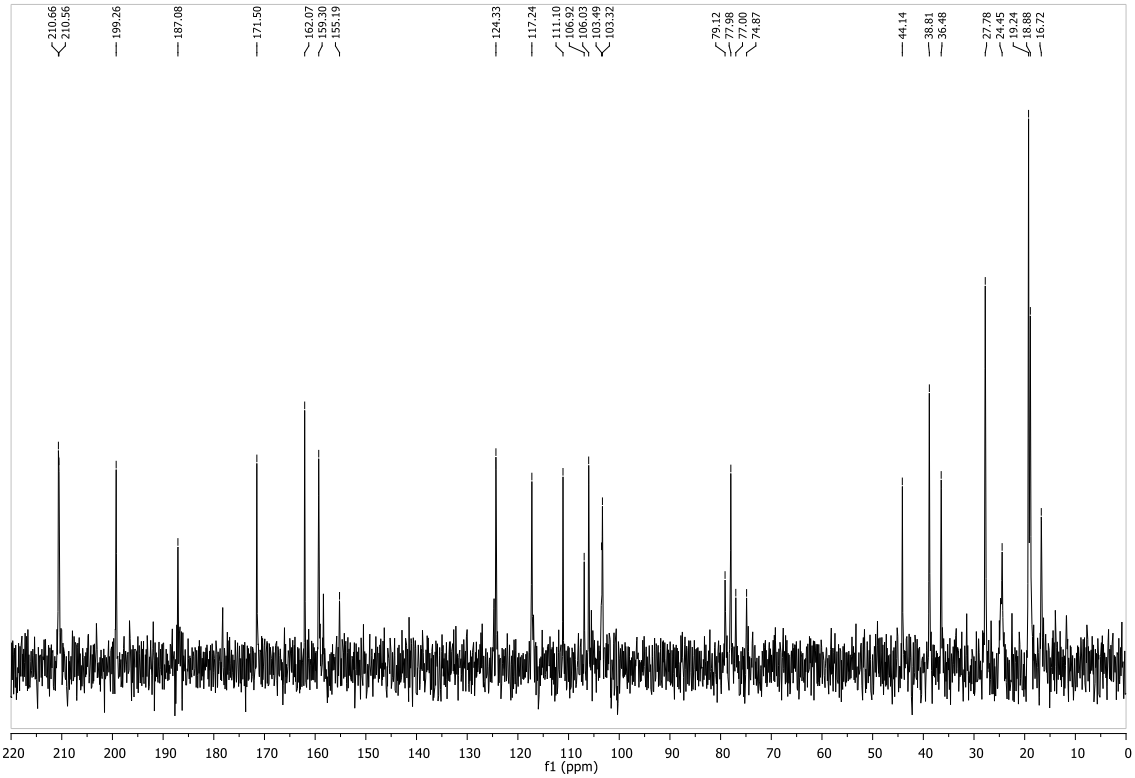

Figura 2.3 Espectro de RMN-^{13}C de **uliginosina B** em $CDCl_3$ a 15 MHz

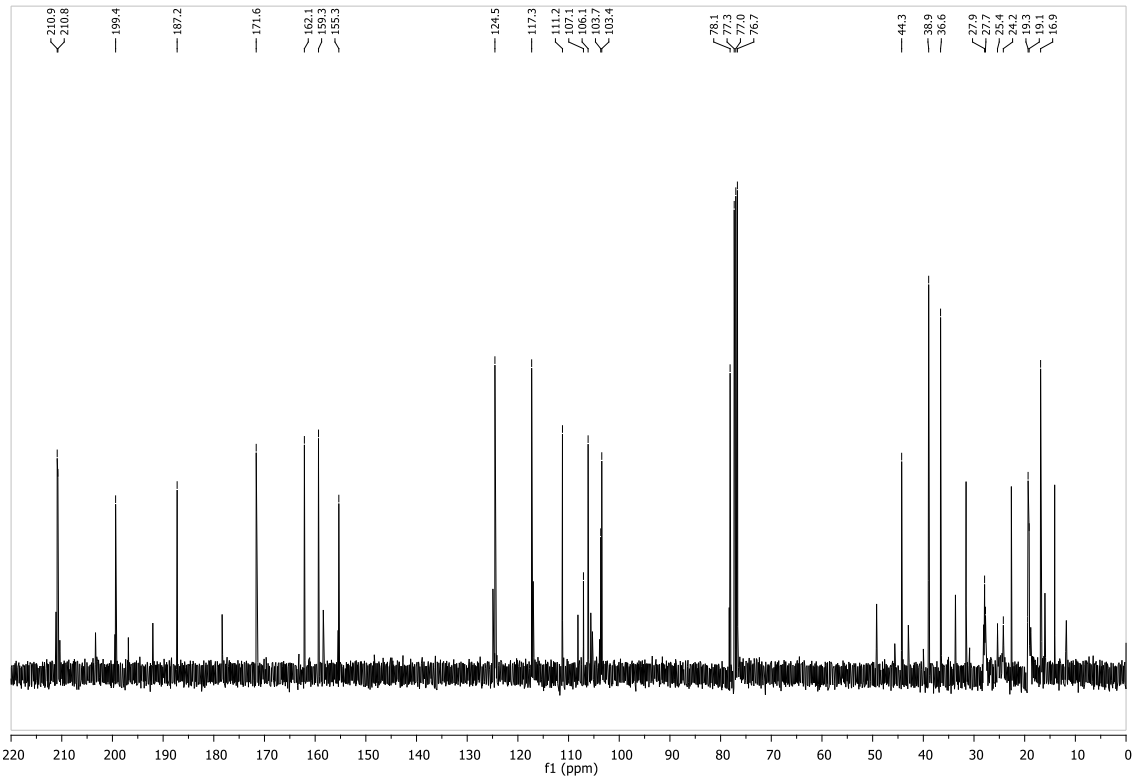

Figura 2.4 Espectro de RMN-^{13}C de **uliginosina B** em $CDCl_3$ a 100 MHz

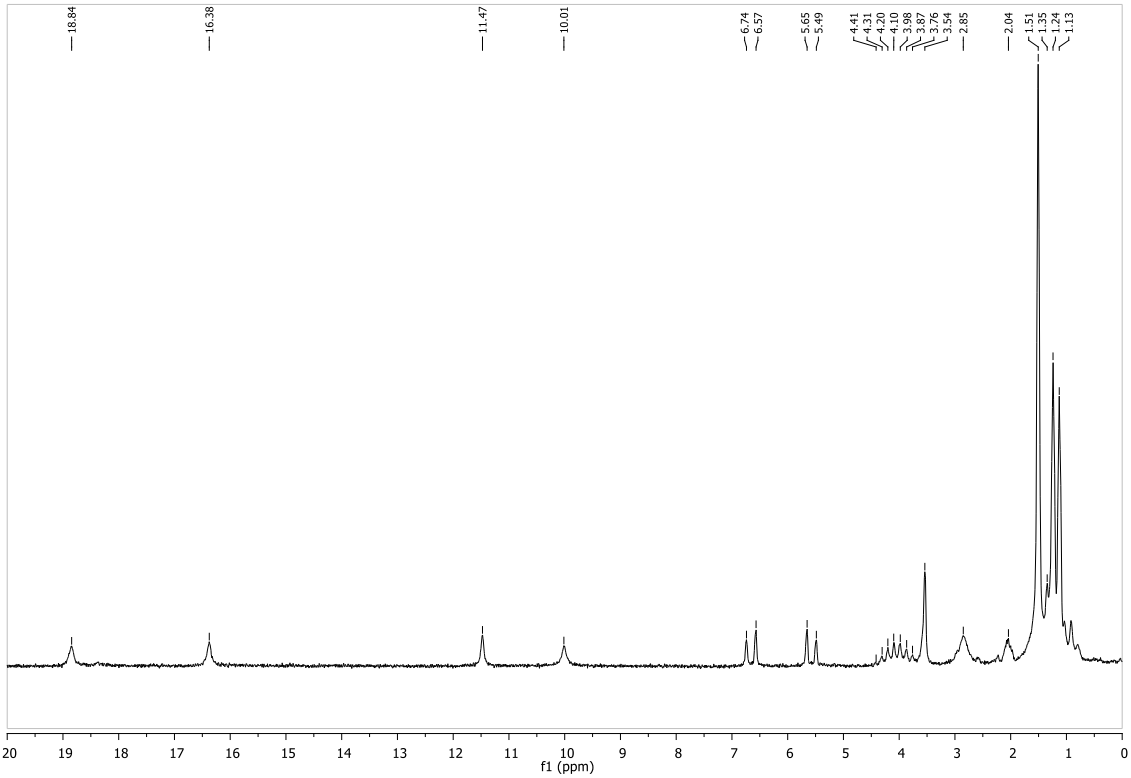

Figura 2.5 Espectro de RMN-^{1}H de **uliginosina B** em acetona-d_6 a 60 MHz

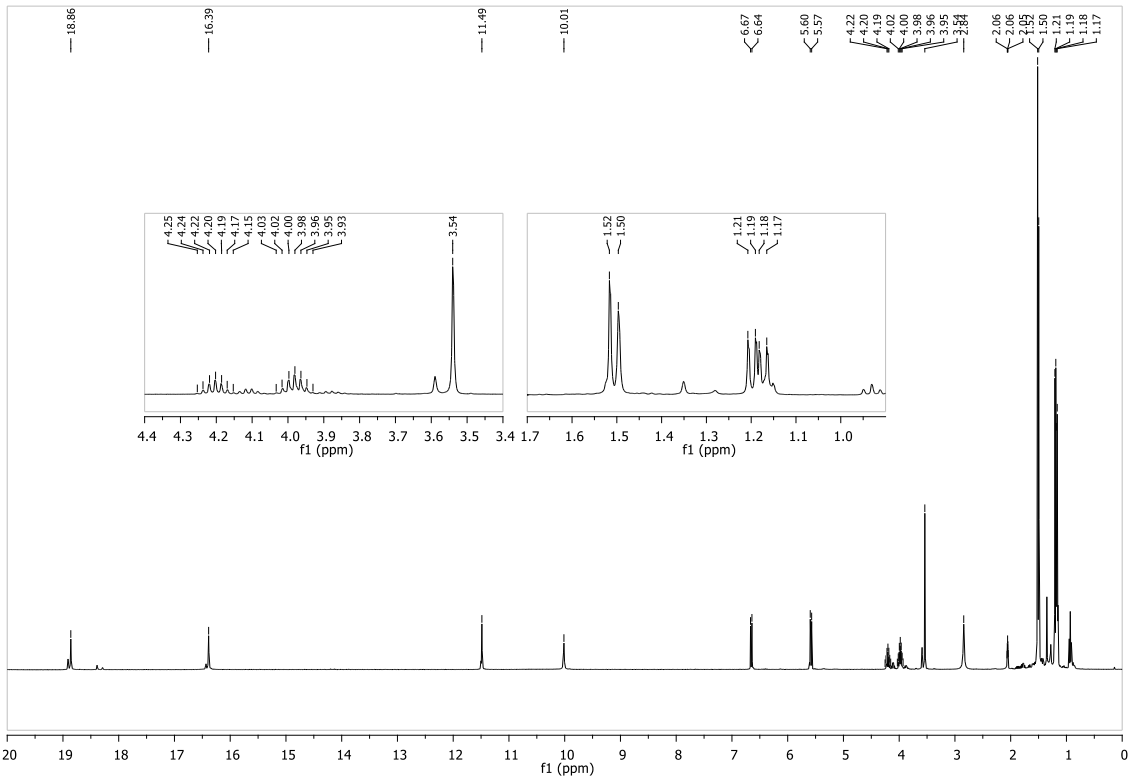

Figura 2.6 Espectro de RMN-^{1}H de **uliginosina B** em acetona-d_6 a 400 MHz

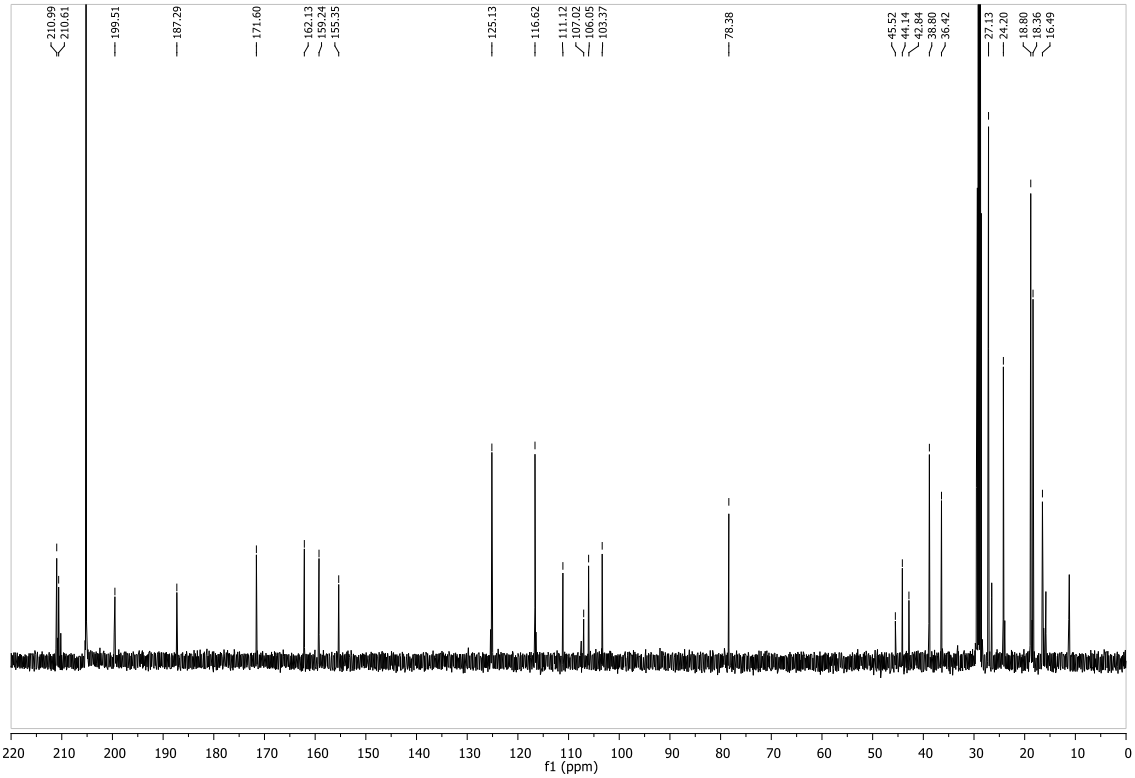

Figura 2.7 Espectro de RMN-^{13}C de **uliginosina B** em acetona-d_6 a 100 MHz

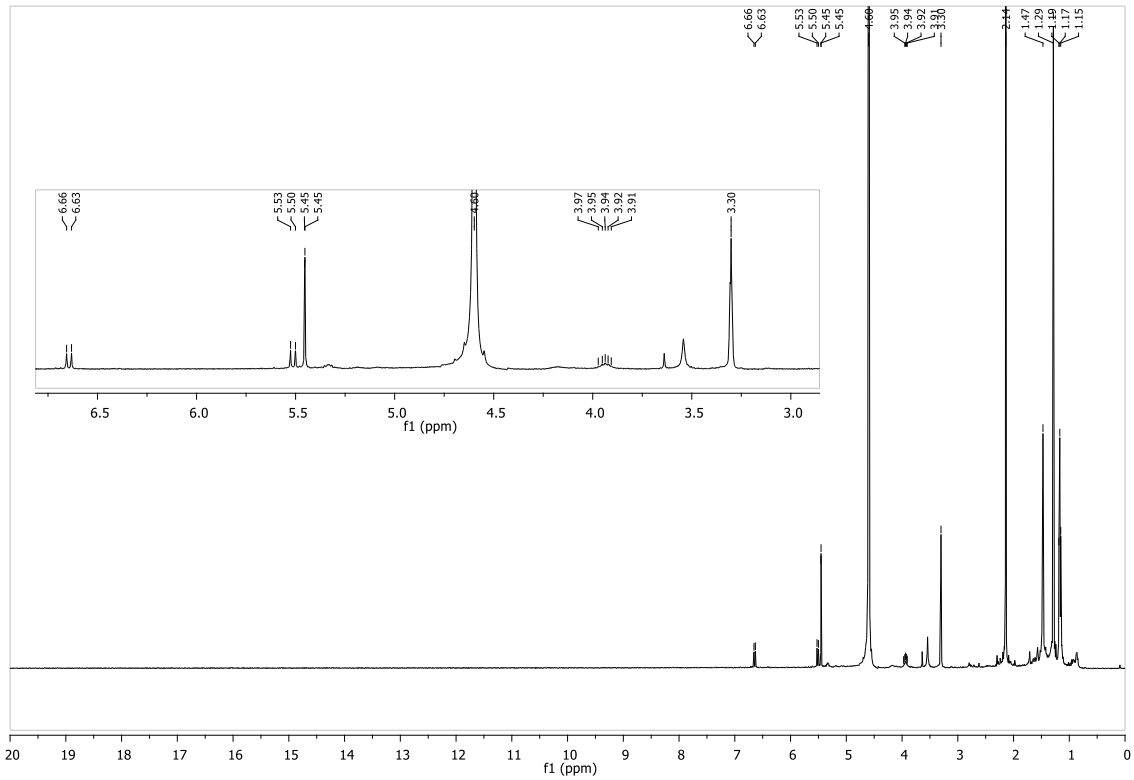

Figura 2.8 Espectro de RMN-^{1}H de **uliginosina B** em CD_3OD a 400 MHz

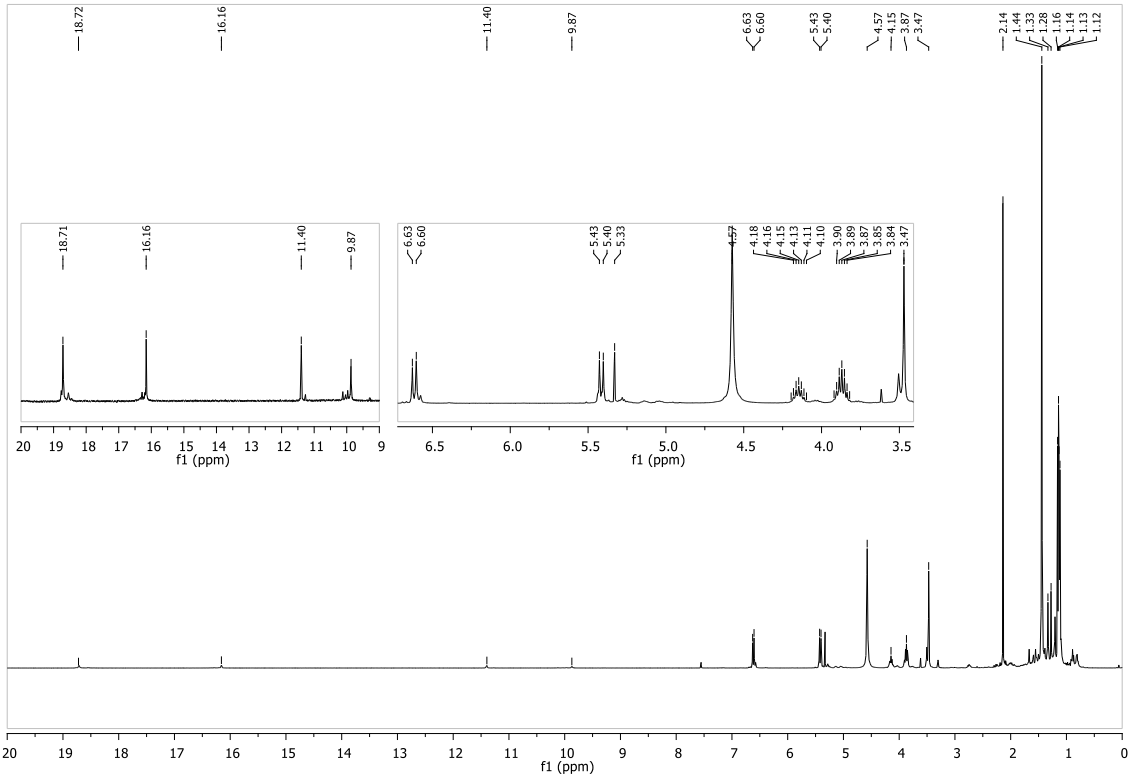

Figura 2.9 Espectro de RMN-^{1}H de **uliginosina B** em CD_3OD + $CDCl_3$ (1:1) a 400 MHz

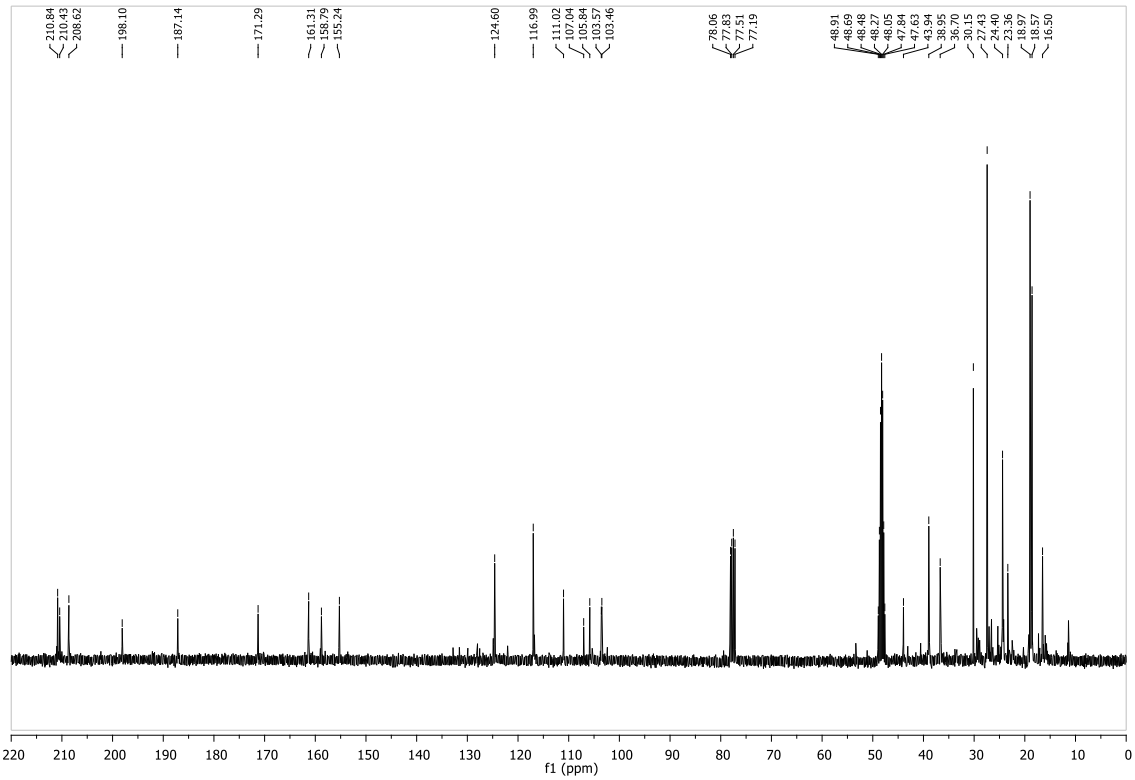

Figura 2.10 Espectro de RMN-^{13}C de **uliginosina B** em CD_3OD + $CDCl_3$ (1:1) a 100 MHz

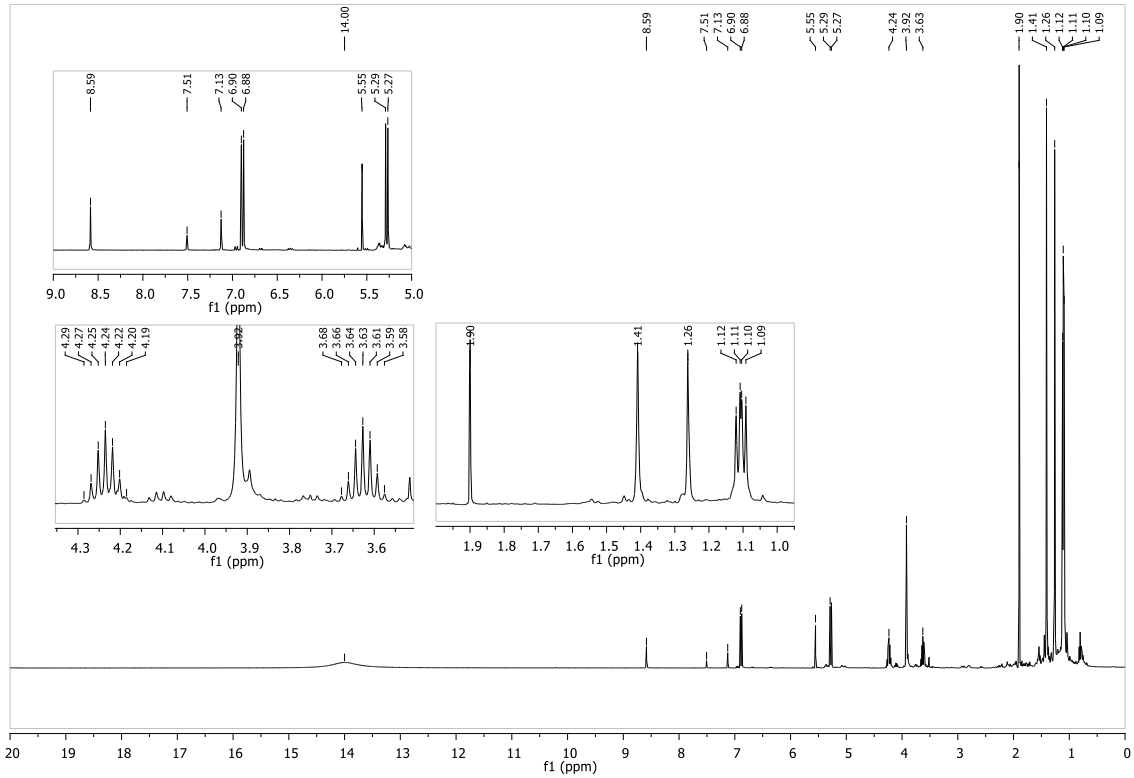

Figura 2.11 Espectro de RMN-^{1}H de **uliginosina B** em C_5D_5N a 400 MHz

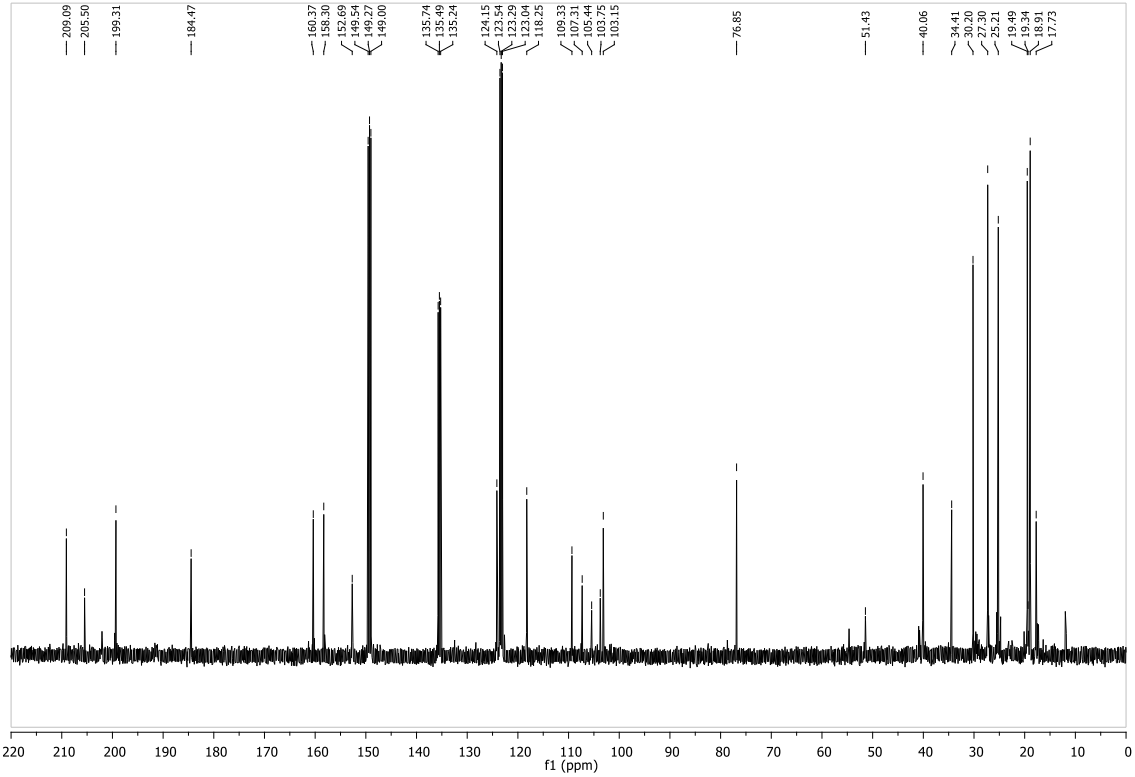

Figura 2.12 Espectro de RMN-^{13}C de **uliginosina B** em C_5D_5N a 100 MHz

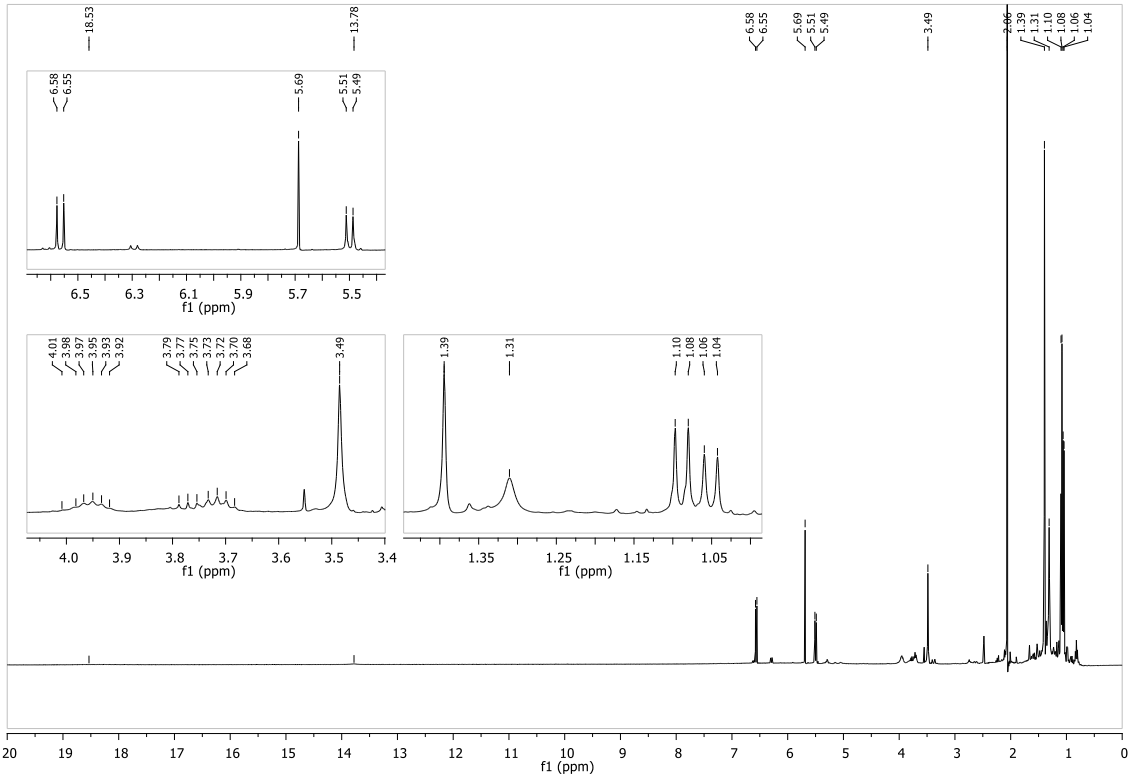

Figura 2.13 Espectro de RMN-^{1}H de **uliginosina B** em CD_3SOCD_3 a 400 MHz

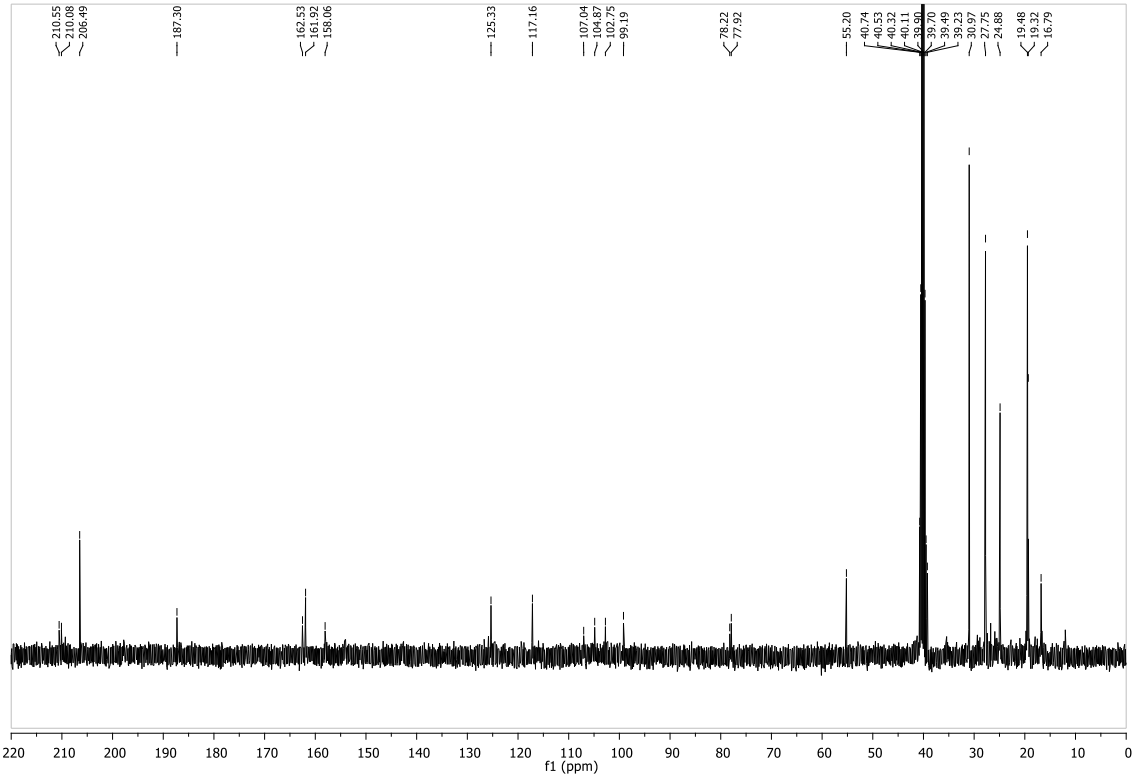

Figura 2.14 Espectro de RMN-^{13}C de **uliginosina B** em CD_3SOCD_3 a 100 MHz

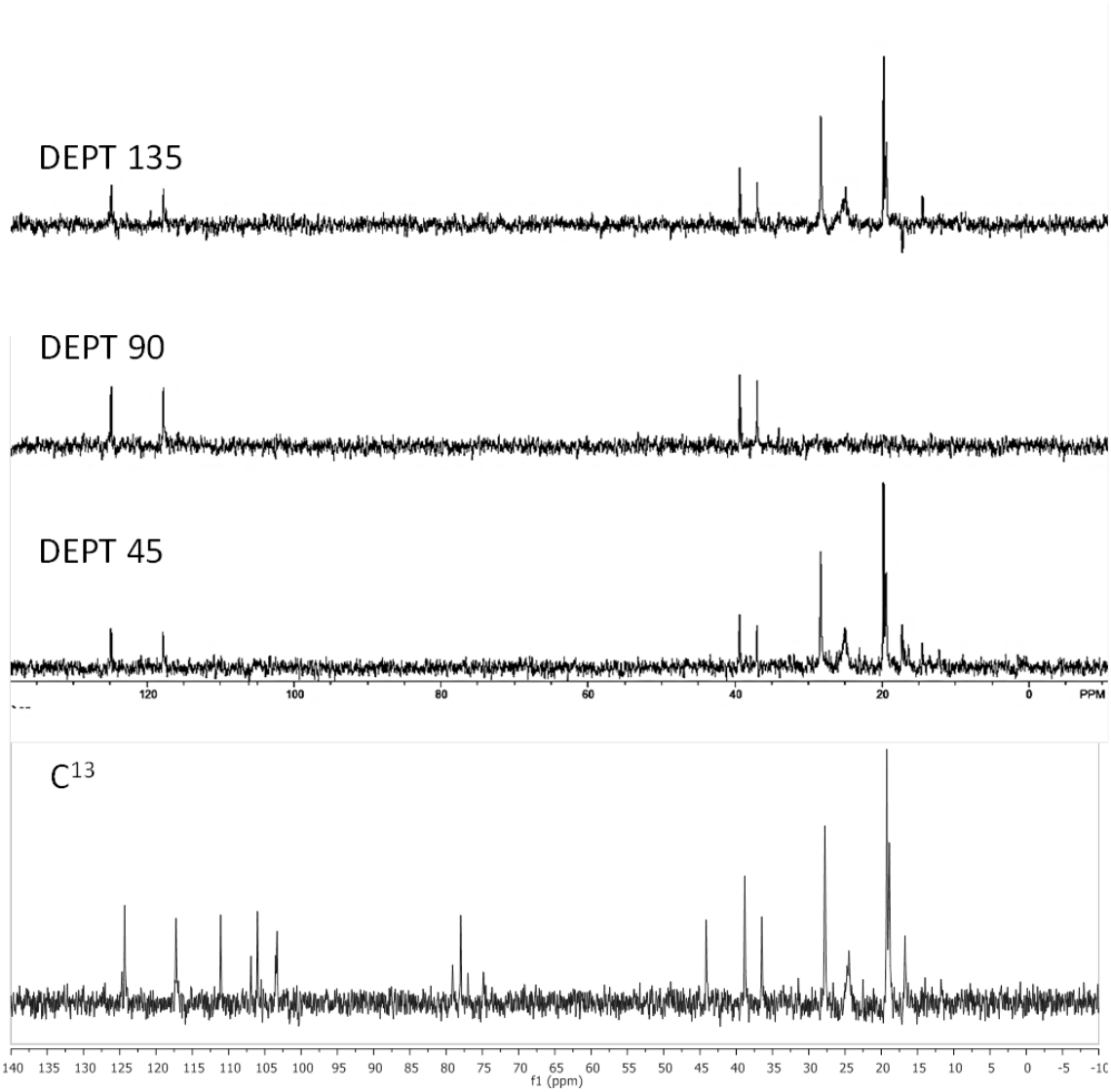

Figura 2.15 Espectro de RMN-^{13}C-DEPT de **uliginosina B** em $CDCl_3$ a 15 MHz

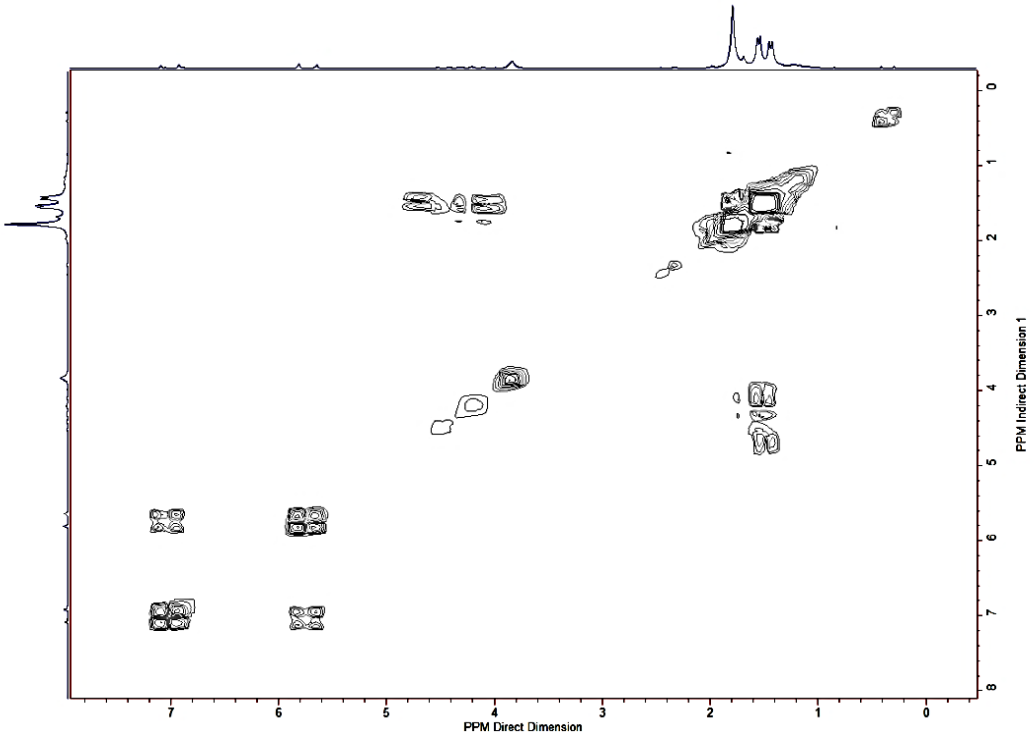

Figura 2.16 Espectro de correlação H-H COSY de **uliginosina B** em $CDCl_3$

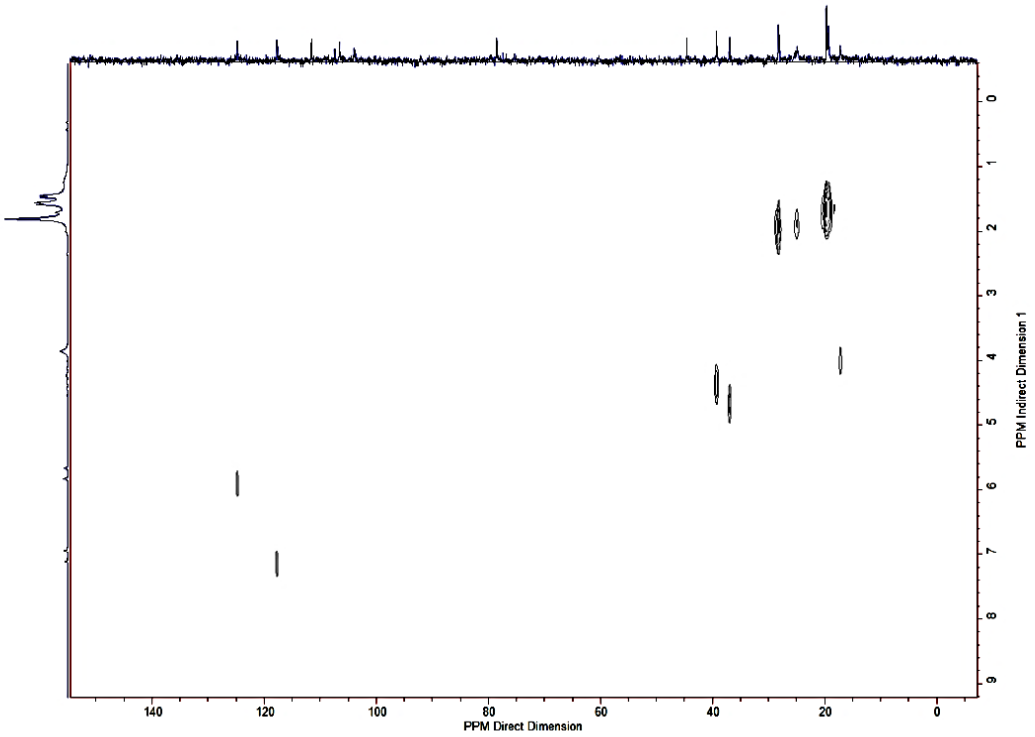

Figura 2.17 Espectro de correlação H-C HETCOR de **uliginosina B** em $CDCl_3$

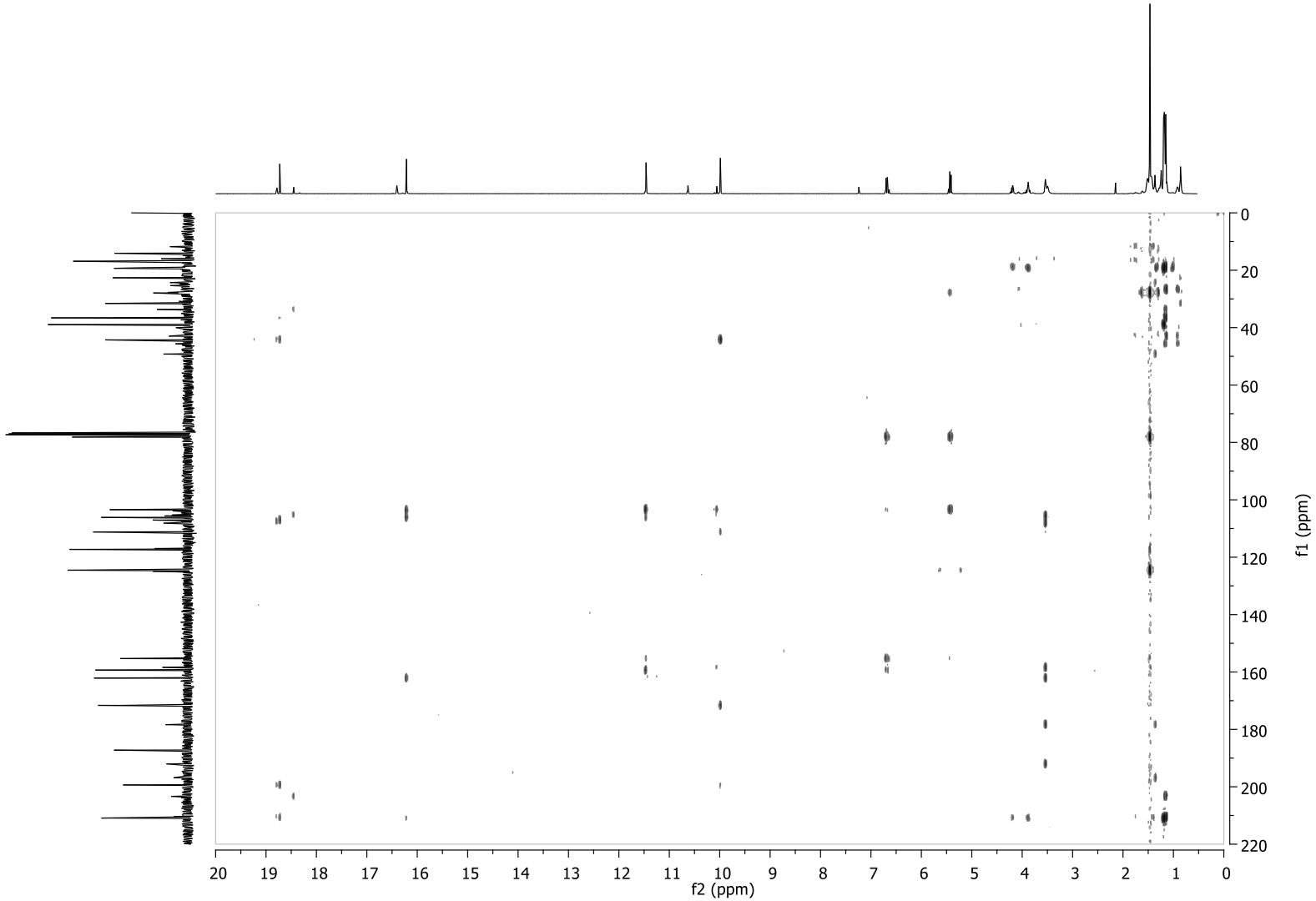

Figura 2.18 Espectro de correlação de acoplamento heteronuclear HMBC de **uliginosina B** em $CDCl_3$ a 400/100 MHz

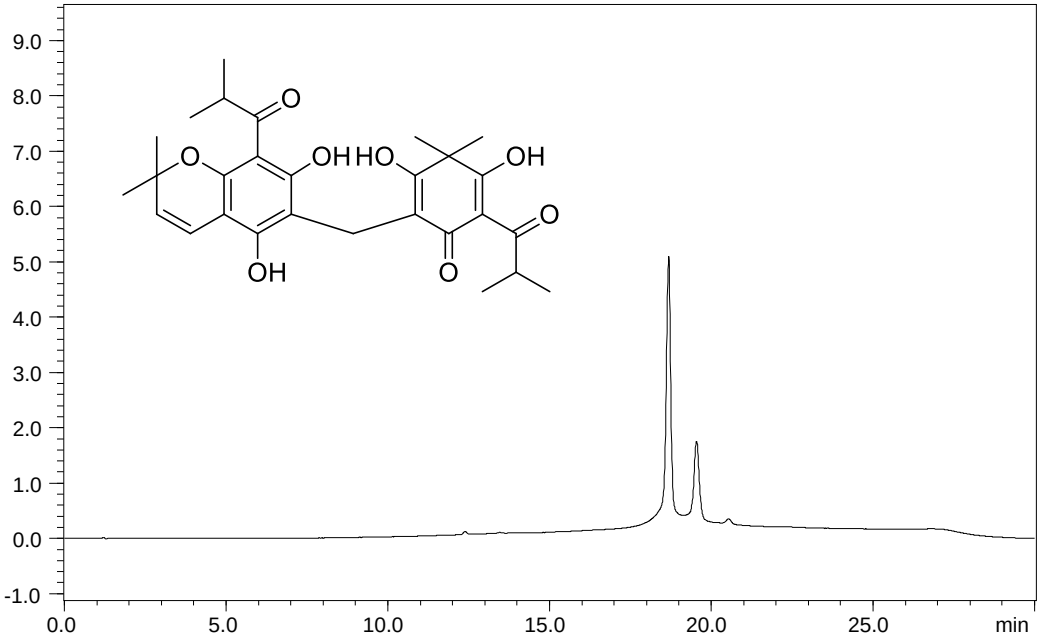

Figure 2.19 Cromatograma por HLPC de uliginosina B

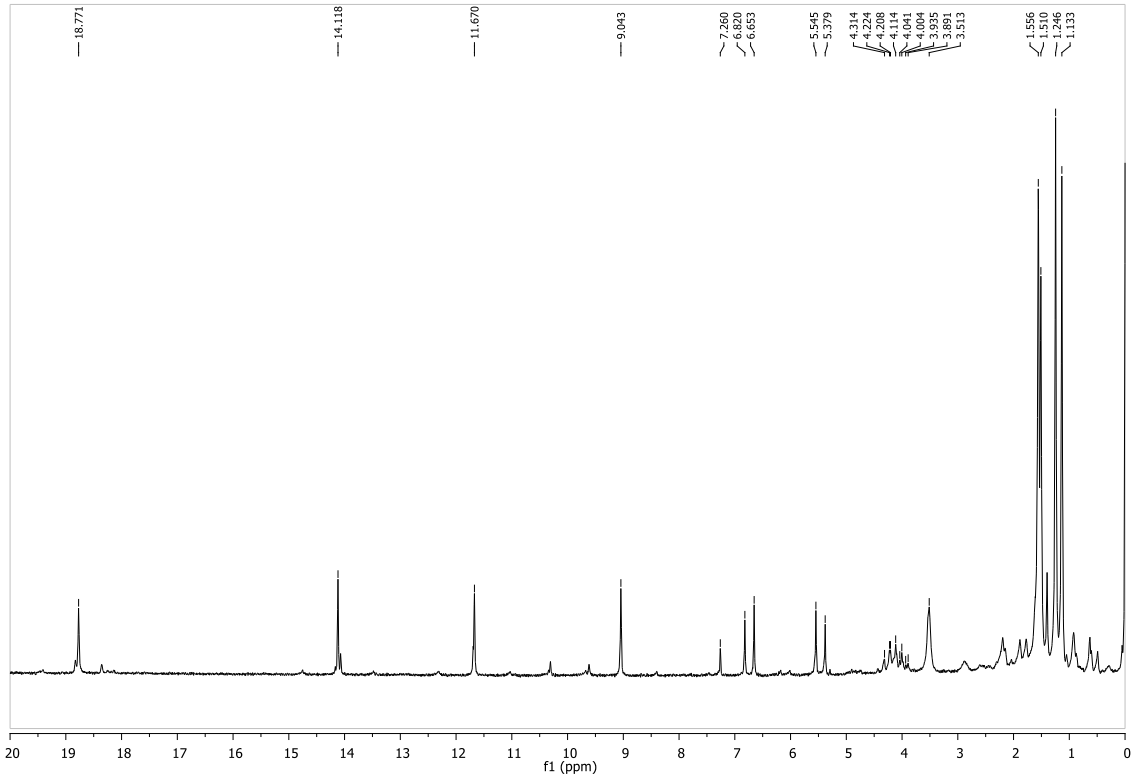

Figura 3.1 Espectro de RMN-^{1}H de **isouliginosina B** em $CDCl_3$ a 60 MHz

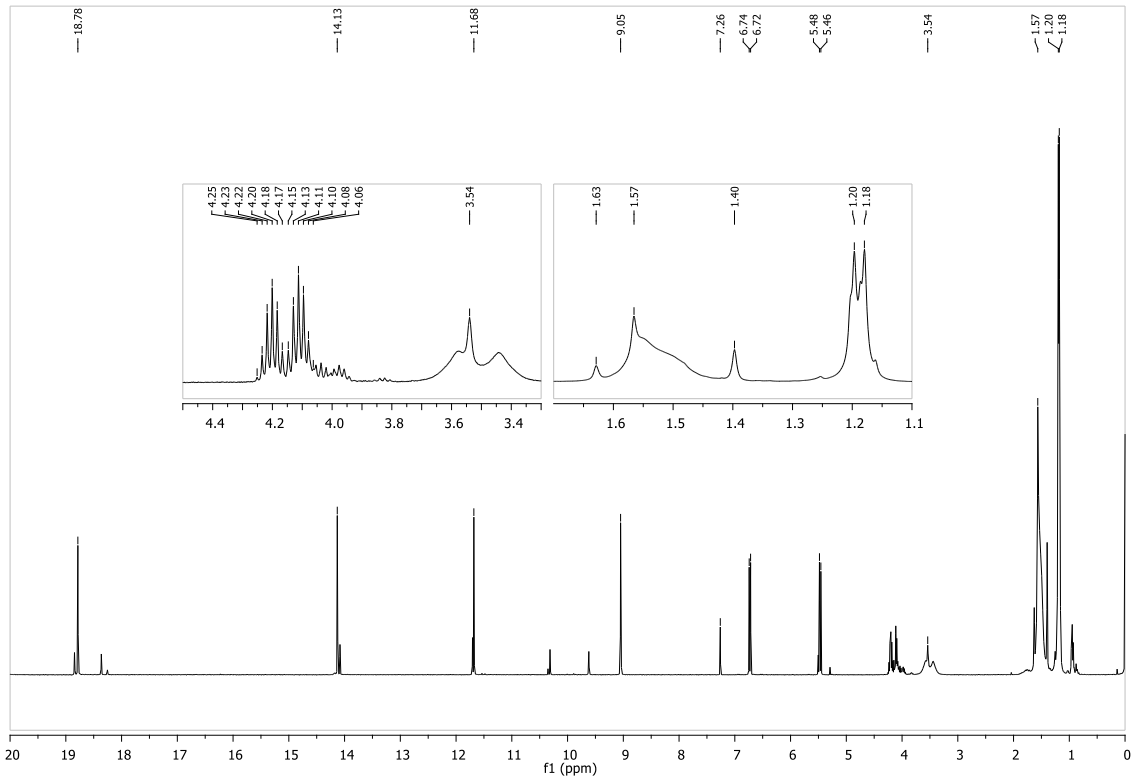

Figura 3.2 Espectro de RMN-^{1}H de **isouliginosina B** em $CDCl_3$ a 400 MHz

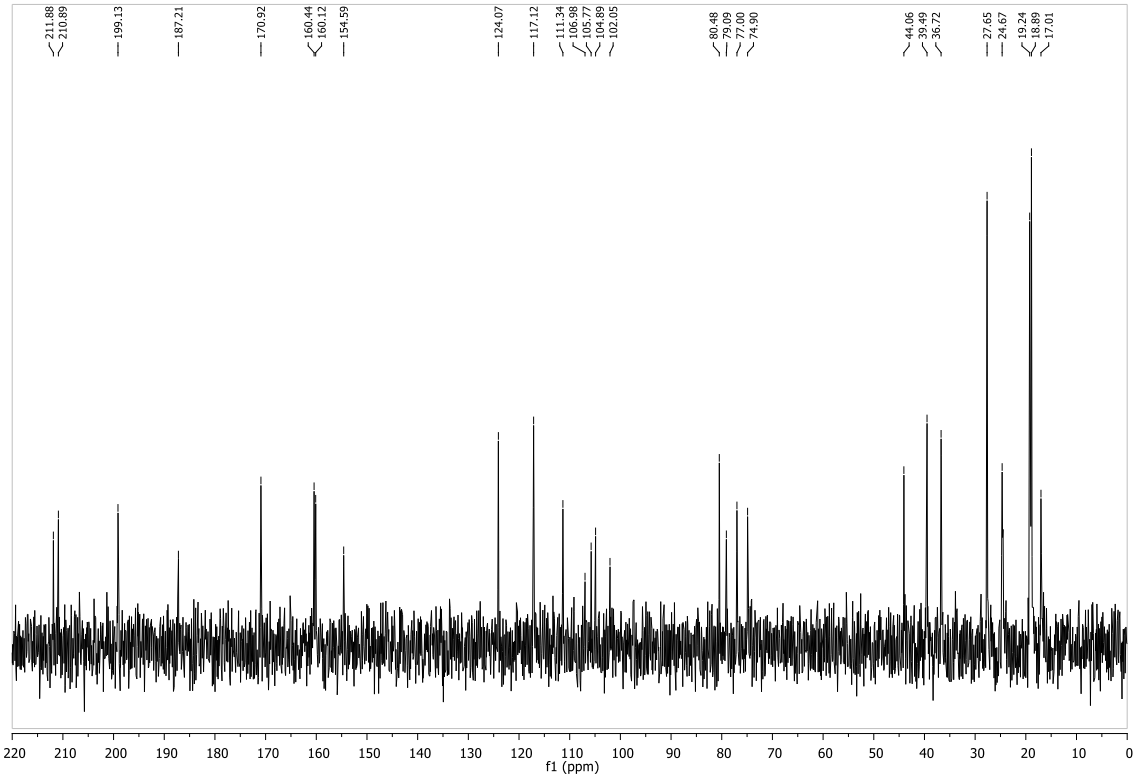

Figura 3.3 Espectro de RMN-^{13}C de **isouliginosina B** em $CDCl_3$ a 15 MHz

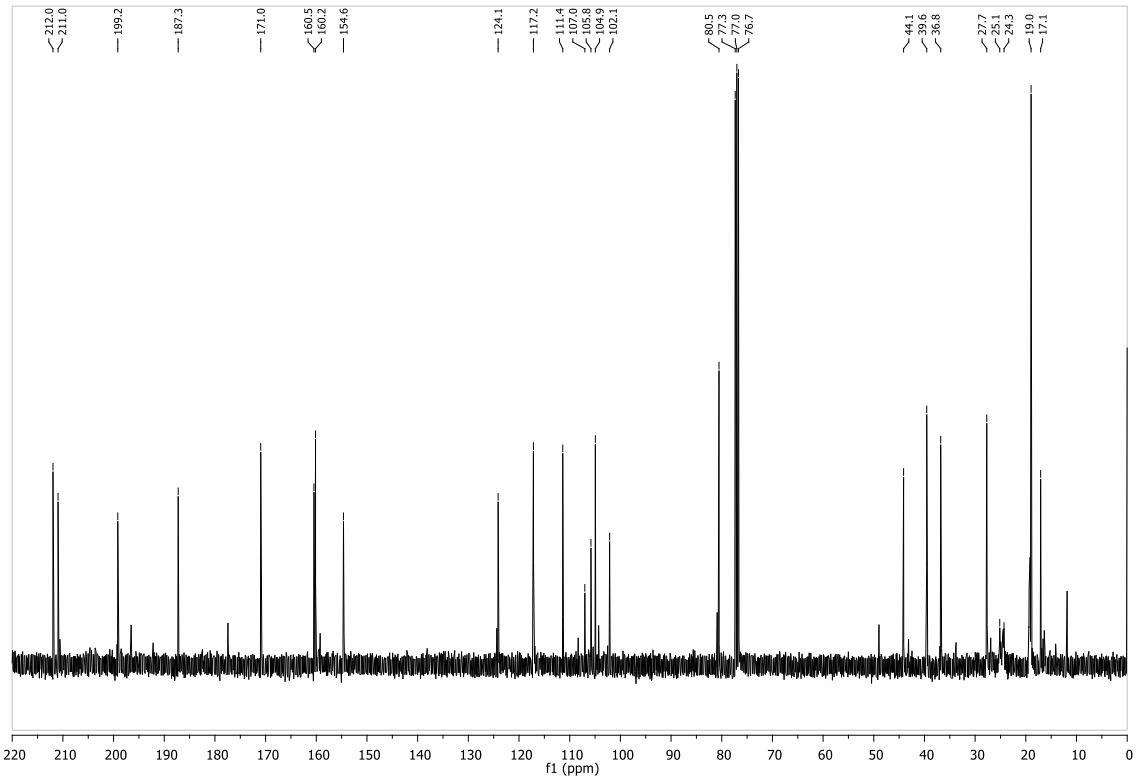

Figura 3.4 Espectro de RMN-^{13}C de **isouliginosina B** em $CDCl_3$ a 100 MHz

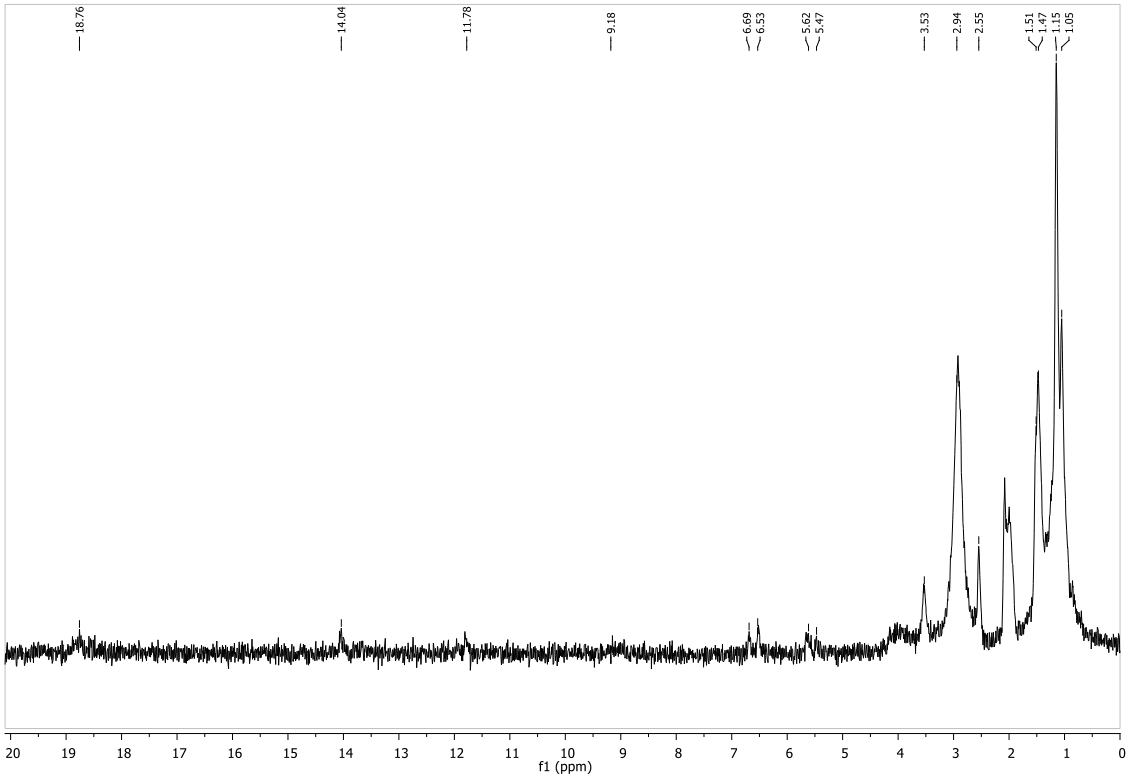

Figura 3.5 Espectro de RMN-^{1}H de **isouliginosina B** em acetona-d_6 a 60 MHz

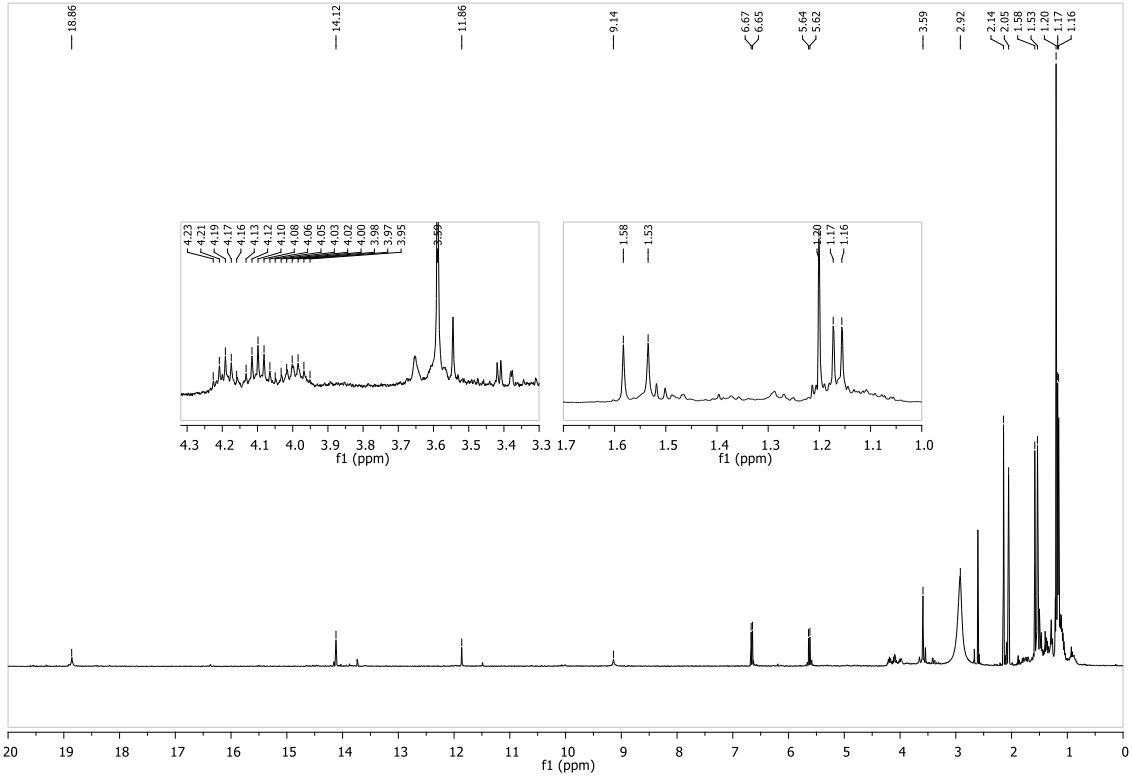

Figura 3.6 Espectro de RMN-^{1}H de **isouliginosina B** em acetona-d_6 a 400 MHz

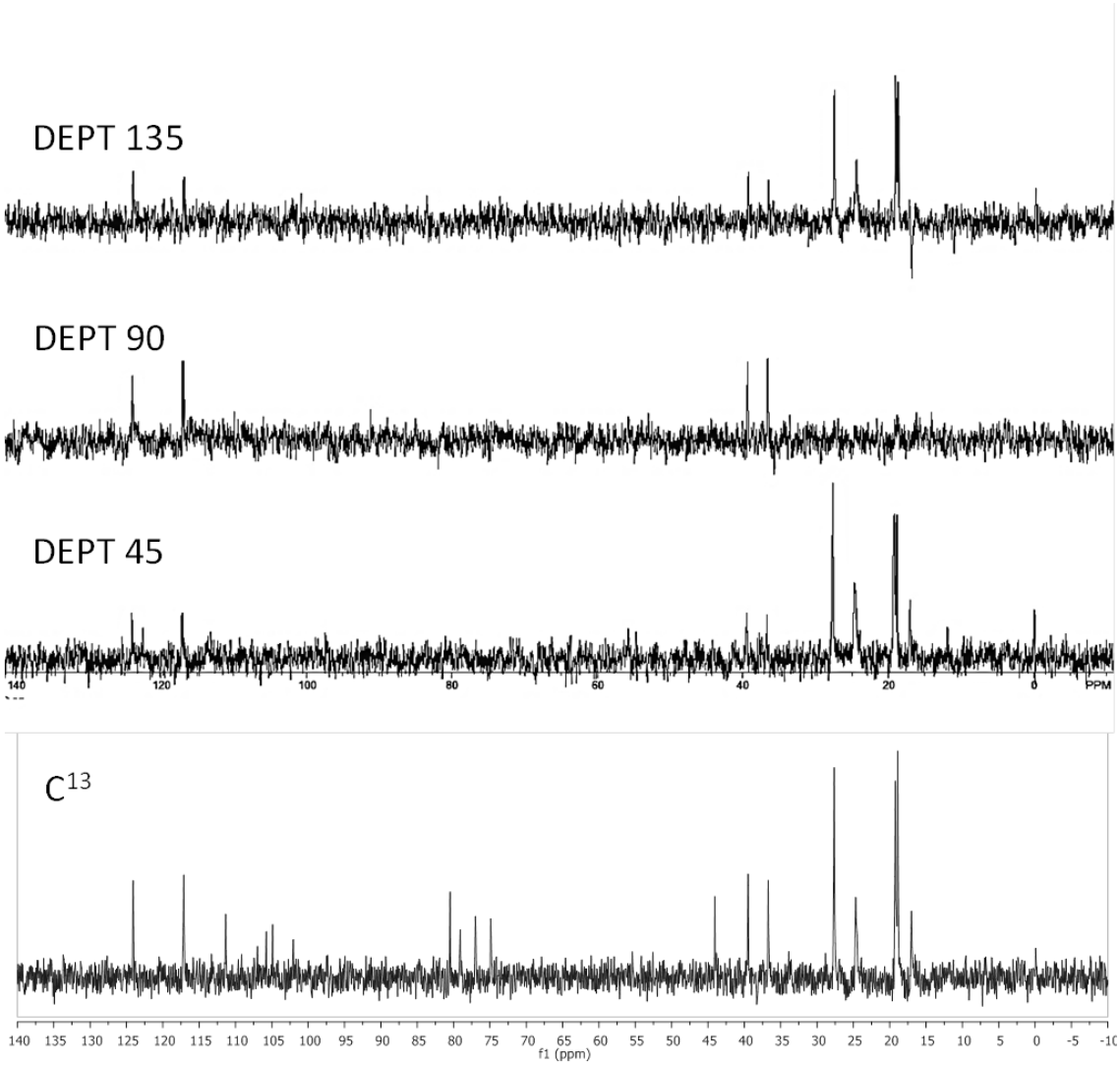

Figura 3.7 Espectro de RMN-^{13}C-DEPT de **isouliginosina B** em $CDCl_3$ a 15 MHz

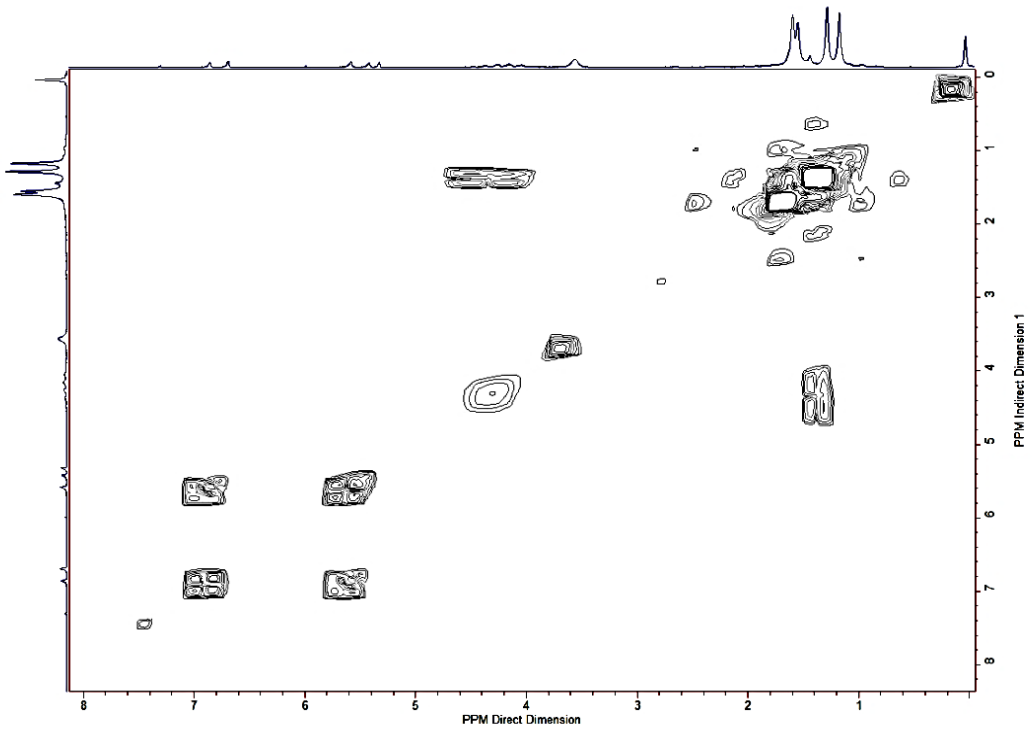

Figura 3.8 Espectro de correlação H-H COSY de **isouliginosina B** em $CDCl_3$

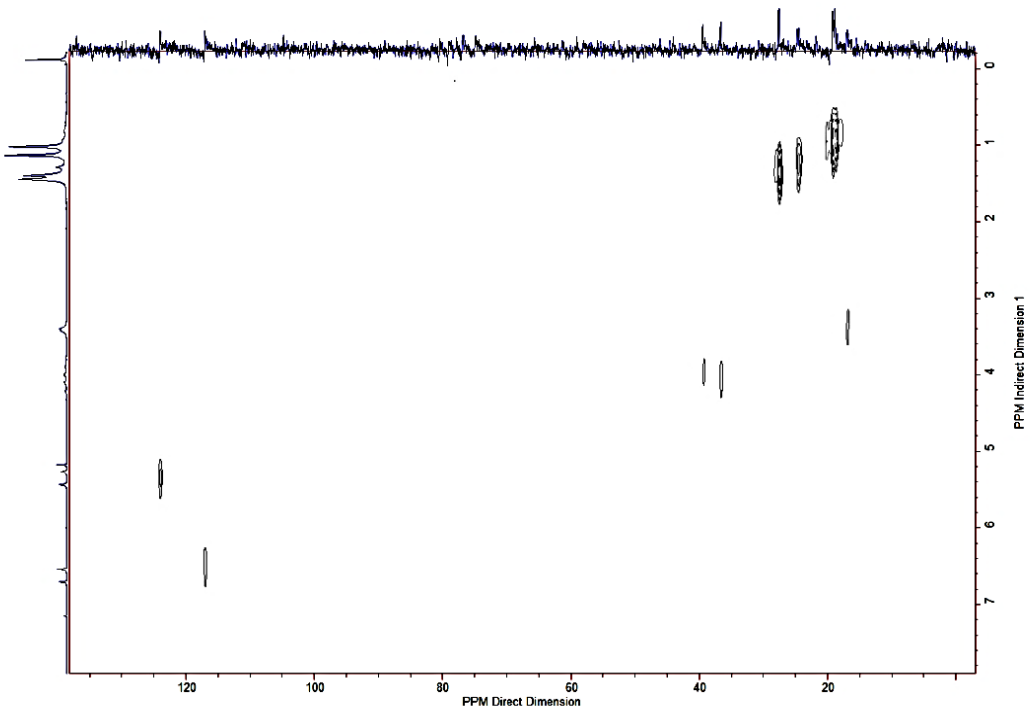

Figura 3.9 Espectro de correlação H-C HETCOR de **isouliginosina B** em $CDCl_3$

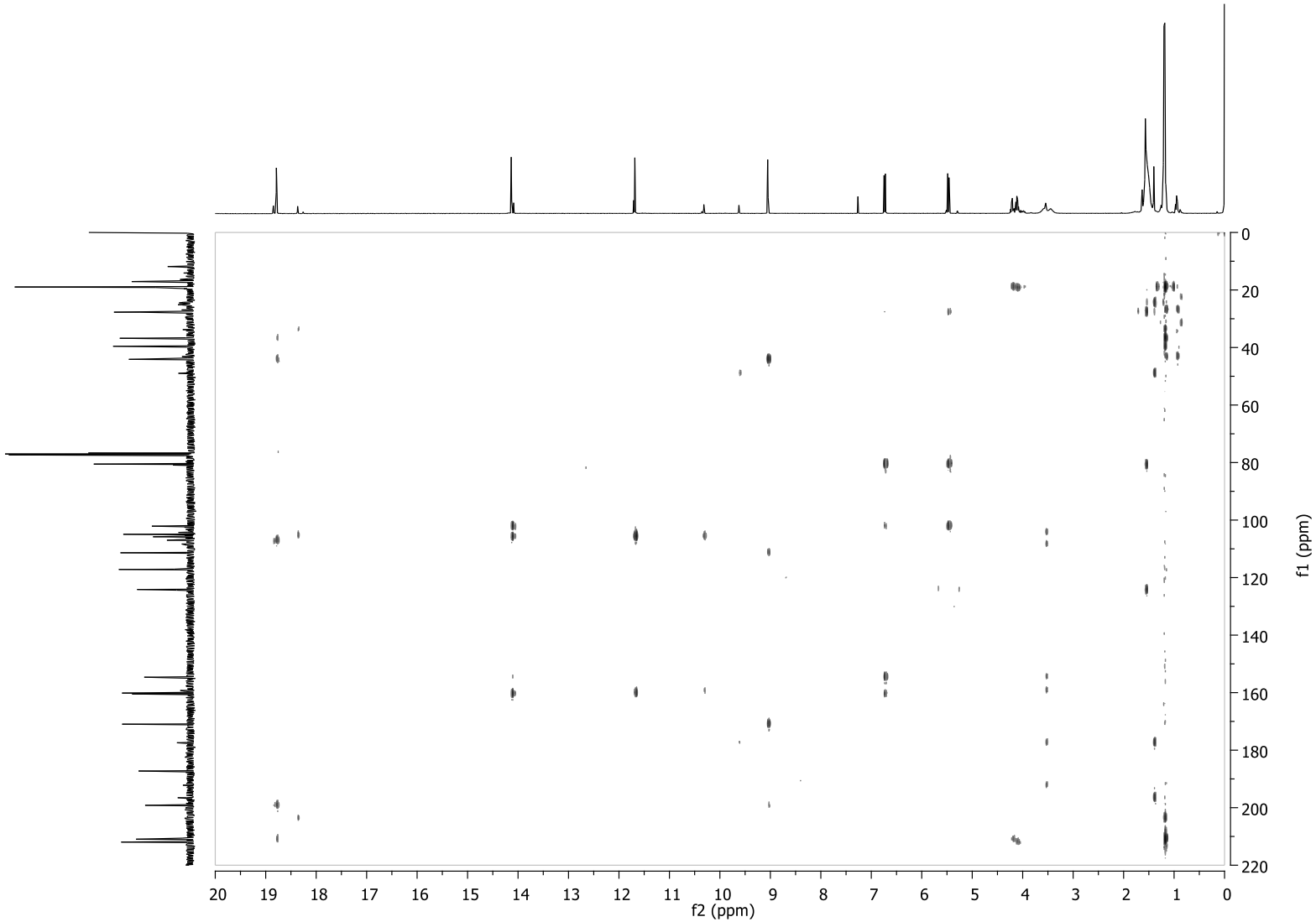

Figura 3.10 Espectro de correlação de acoplamento heteronuclear HMBC de **isouliginosina B** em $CDCl_3$ a 400/100 MHz

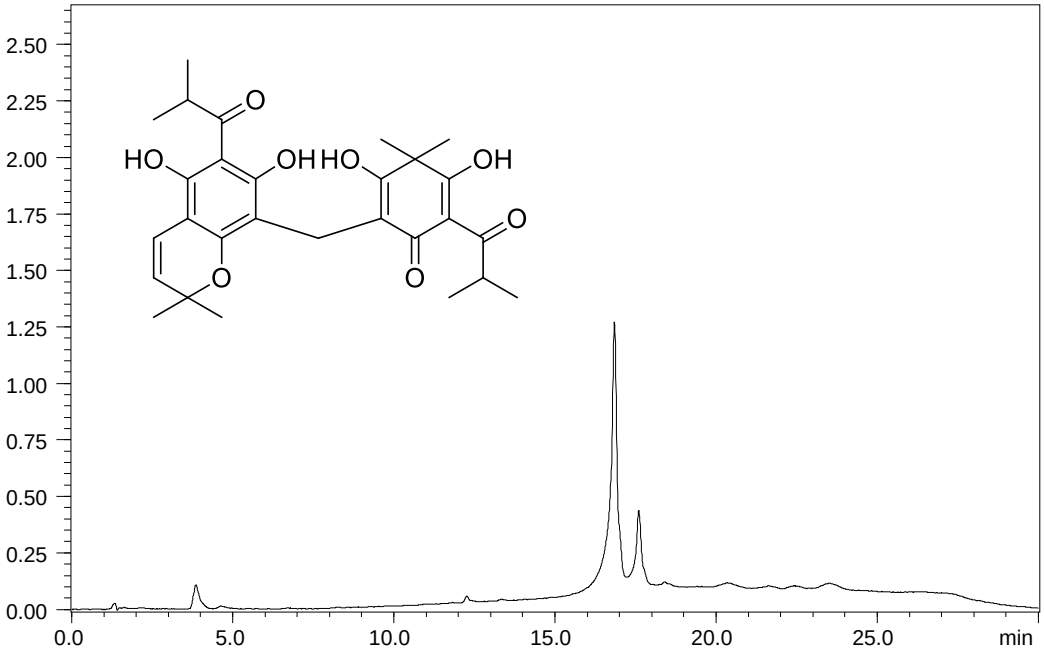

Figure 3.11 Cromatograma por HLPC de isouliginosina B

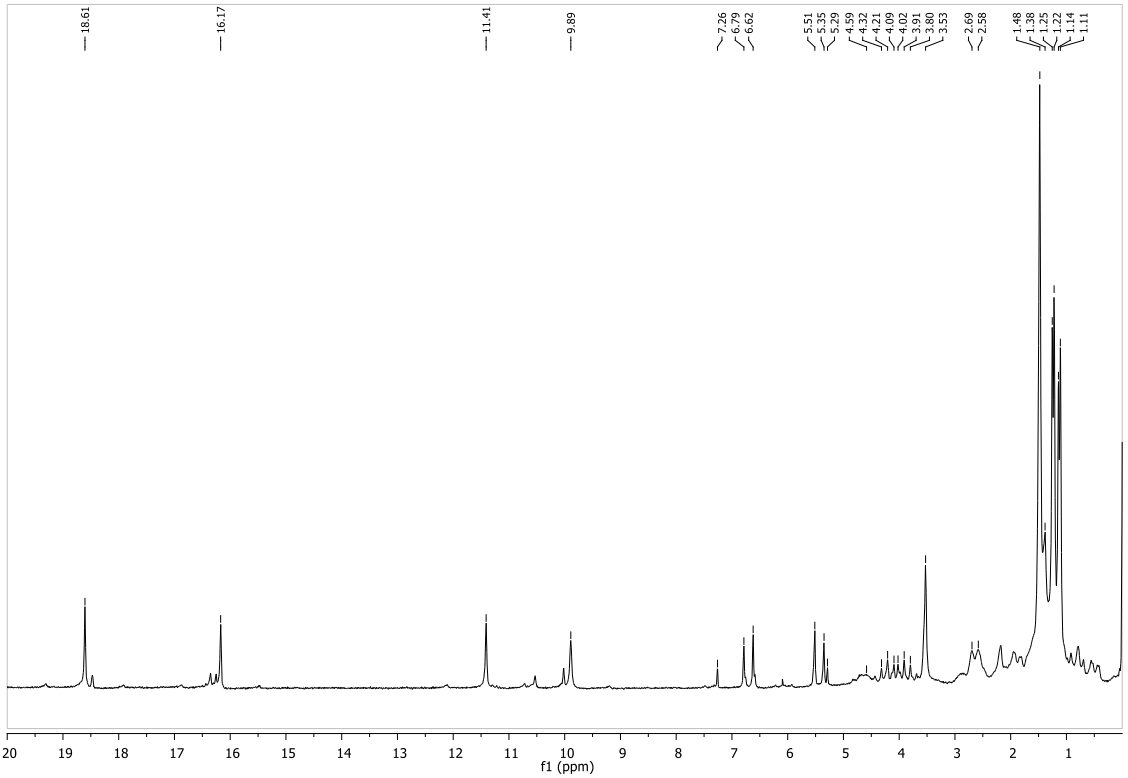

Figura 4.1 Espectro de RMN-^{1}H de **hiperbrasilol B** em $CDCl_3$ a 60 MHz

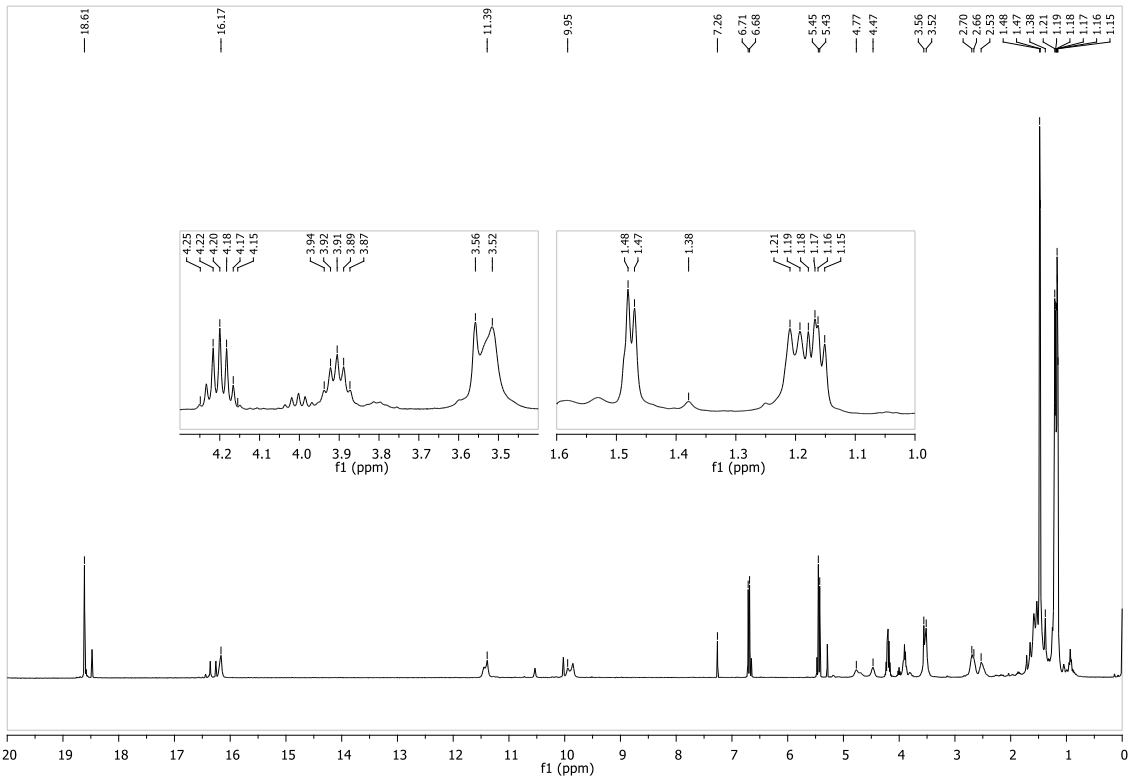

Figura 4.2 Espectro de RMN-^{1}H de **hiperbrasilol B** em $CDCl_3$ a 400 MHz

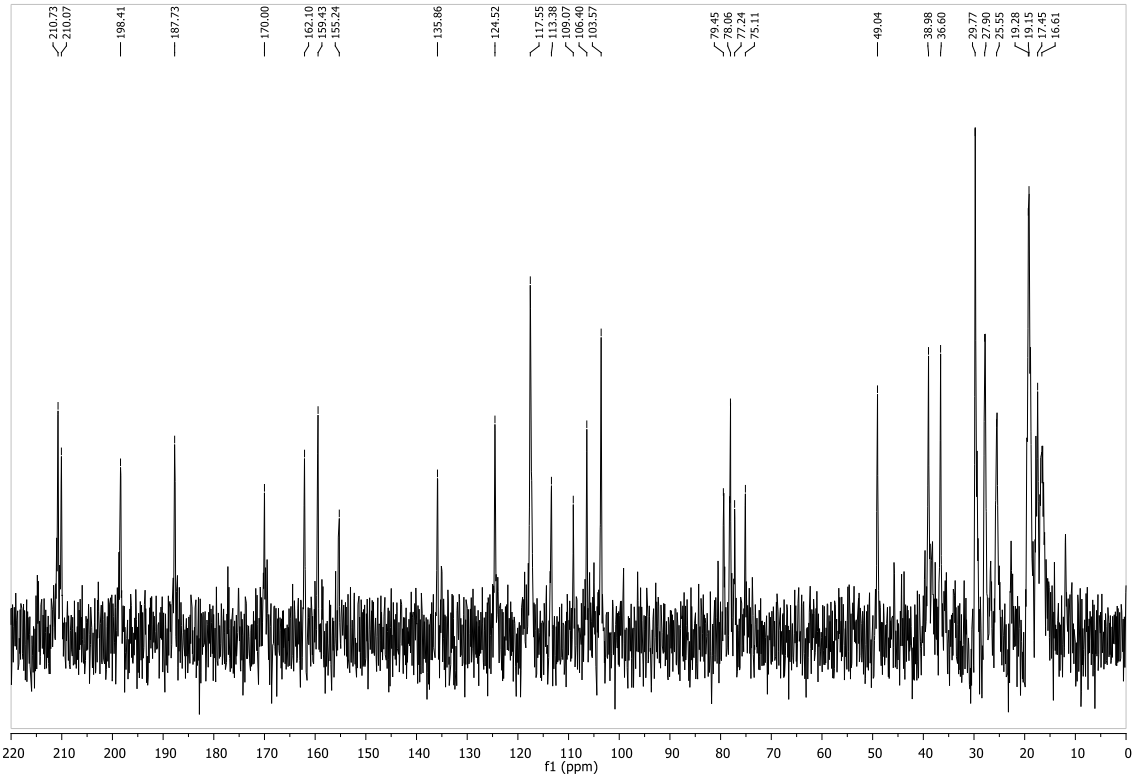

Figura 4.3 Espectro de RMN-^{13}C de **hiperbrasilol B** em $CDCl_3$ a 15 MHz

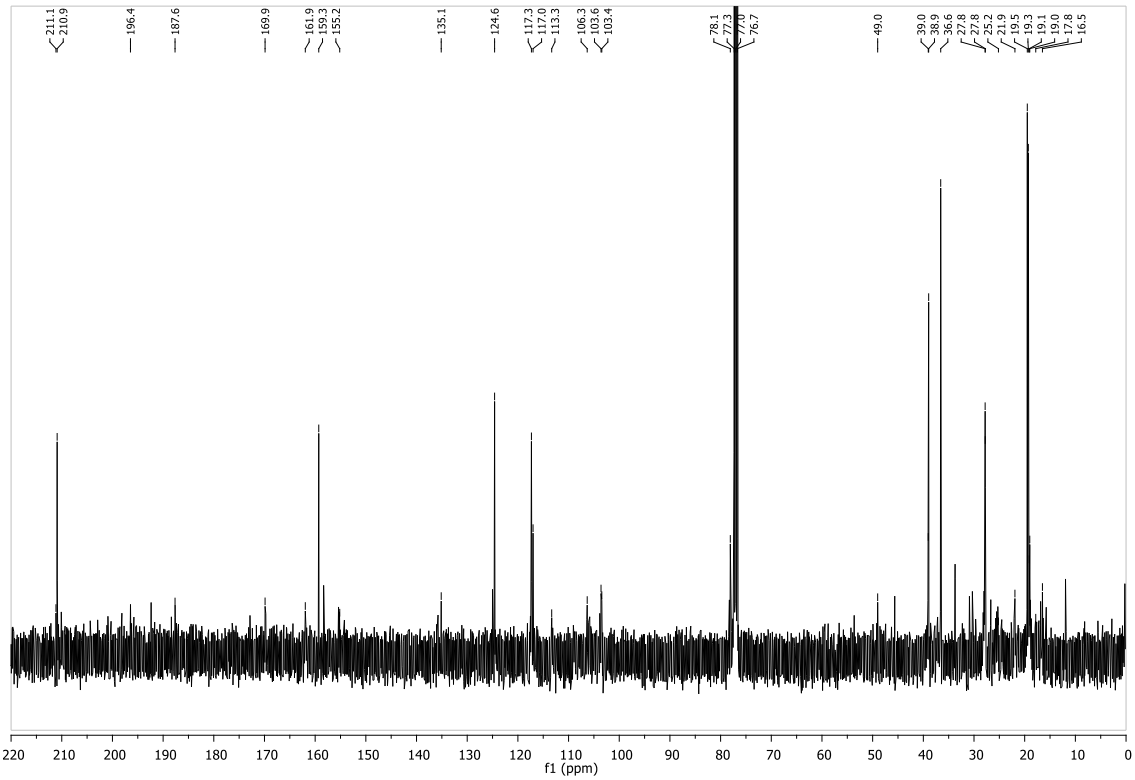

Figura 4.4 Espectro de RMN-^{13}C de **hiperbrasilol B** em $CDCl_3$ a 100 MHz

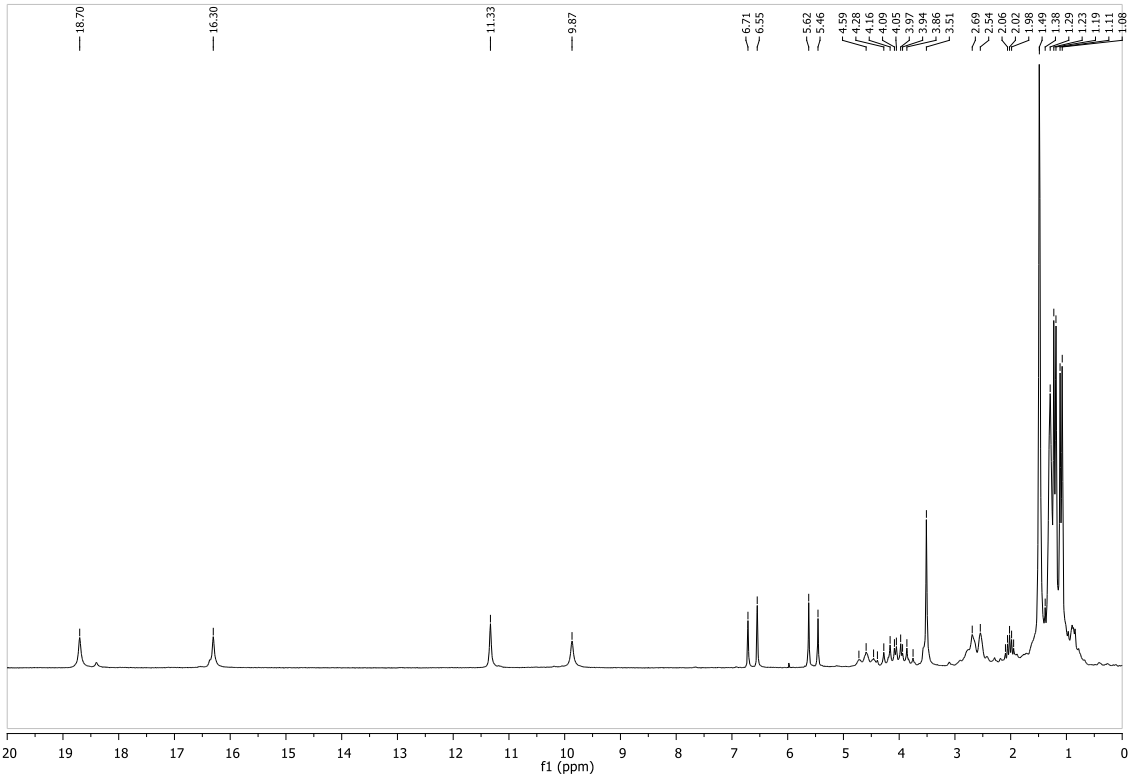

Figura 4.5 Espectro de RMN-^{1}H de **hipebrasilol B** em acetona-d_6 a 60 MHz

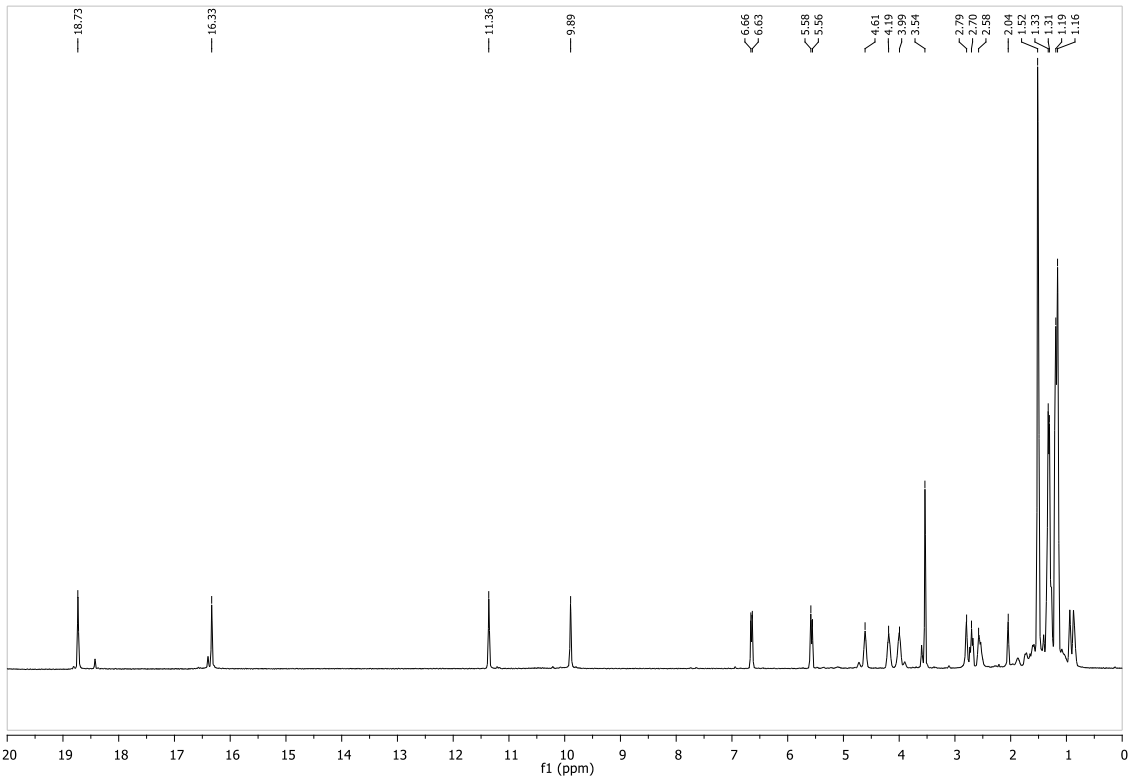

Figura 4.6 Espectro de RMN-^{1}H de **hipebrasilol B** em acetona-d_6 a 400 MHz

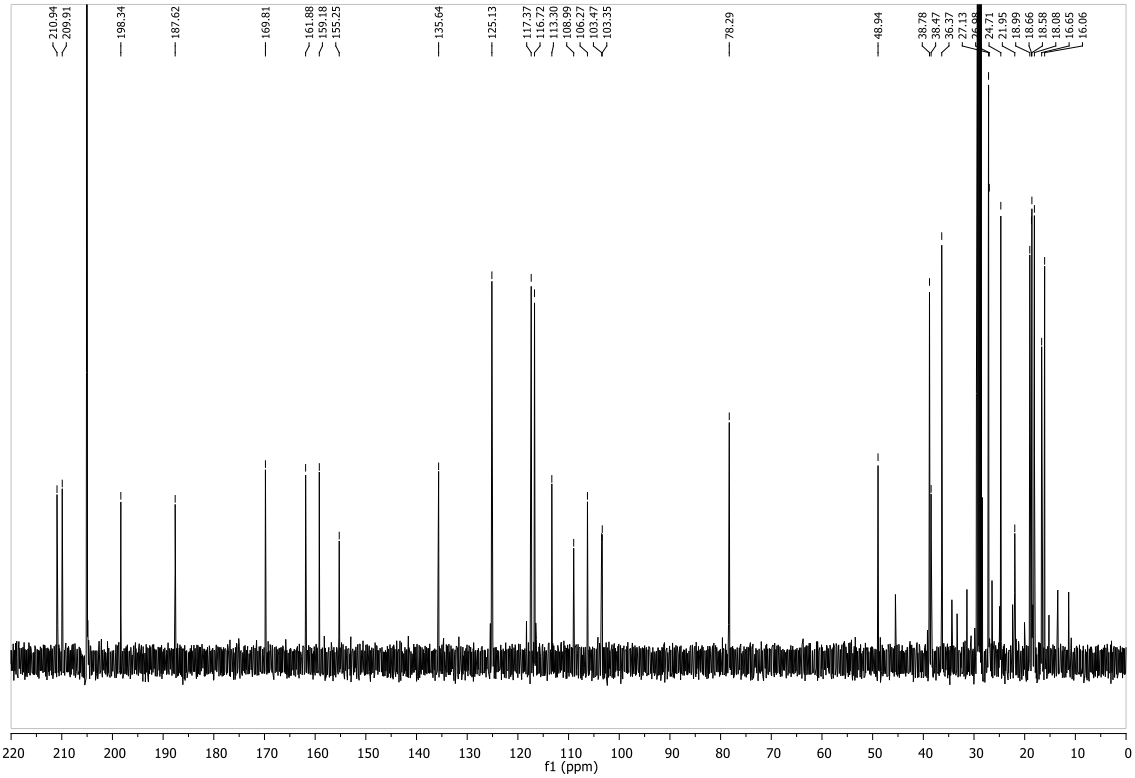

Figura 4.7 Espectro de RMN-^{13}C de **hipebrasilol B** em acetona-d_6 a 100 MHz

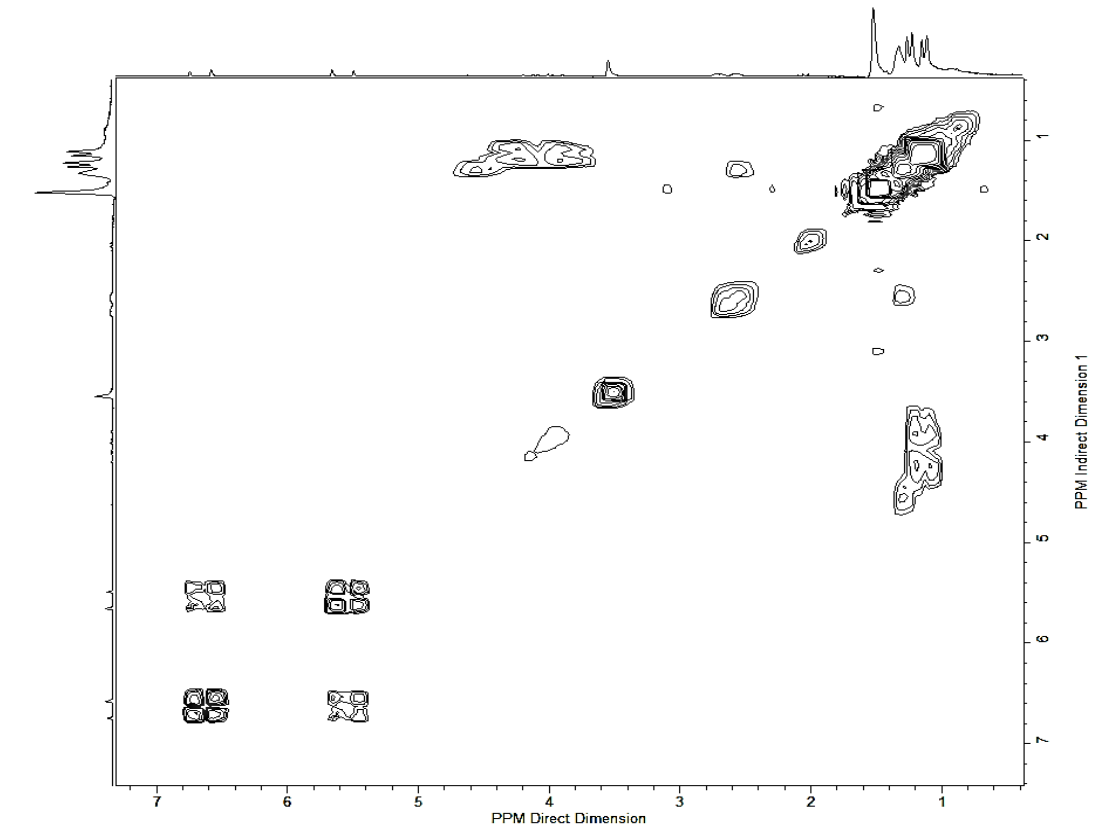

Figura 4.8 Espectro de correlação H-H COSY de **hiperbrasilol B** em $CDCl_3$

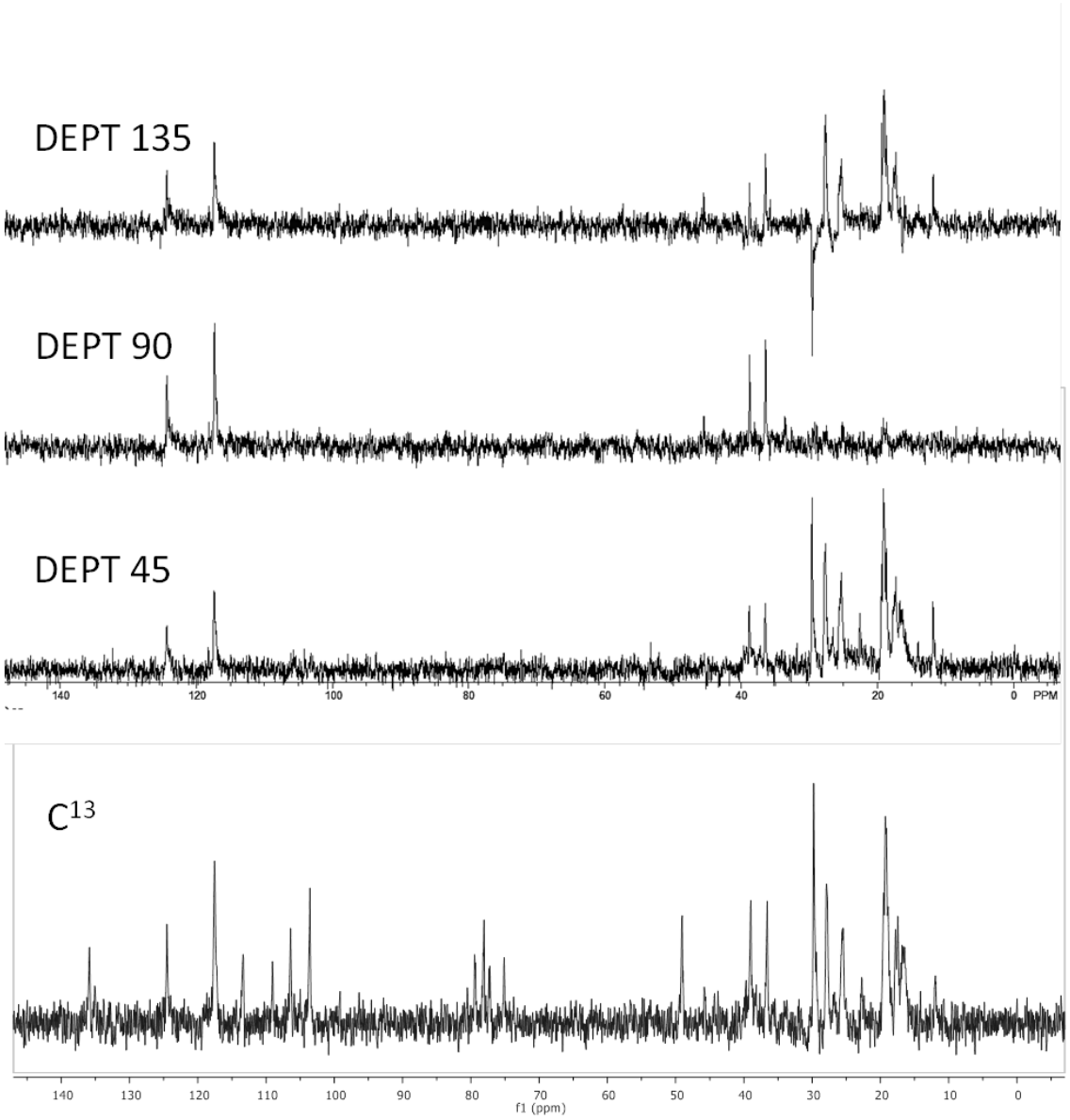

Figura 4.9 Espectro de RMN-^{13}C-DEPT de **hiperbrasilol B** em $CDCl_3$ a 15 MHz

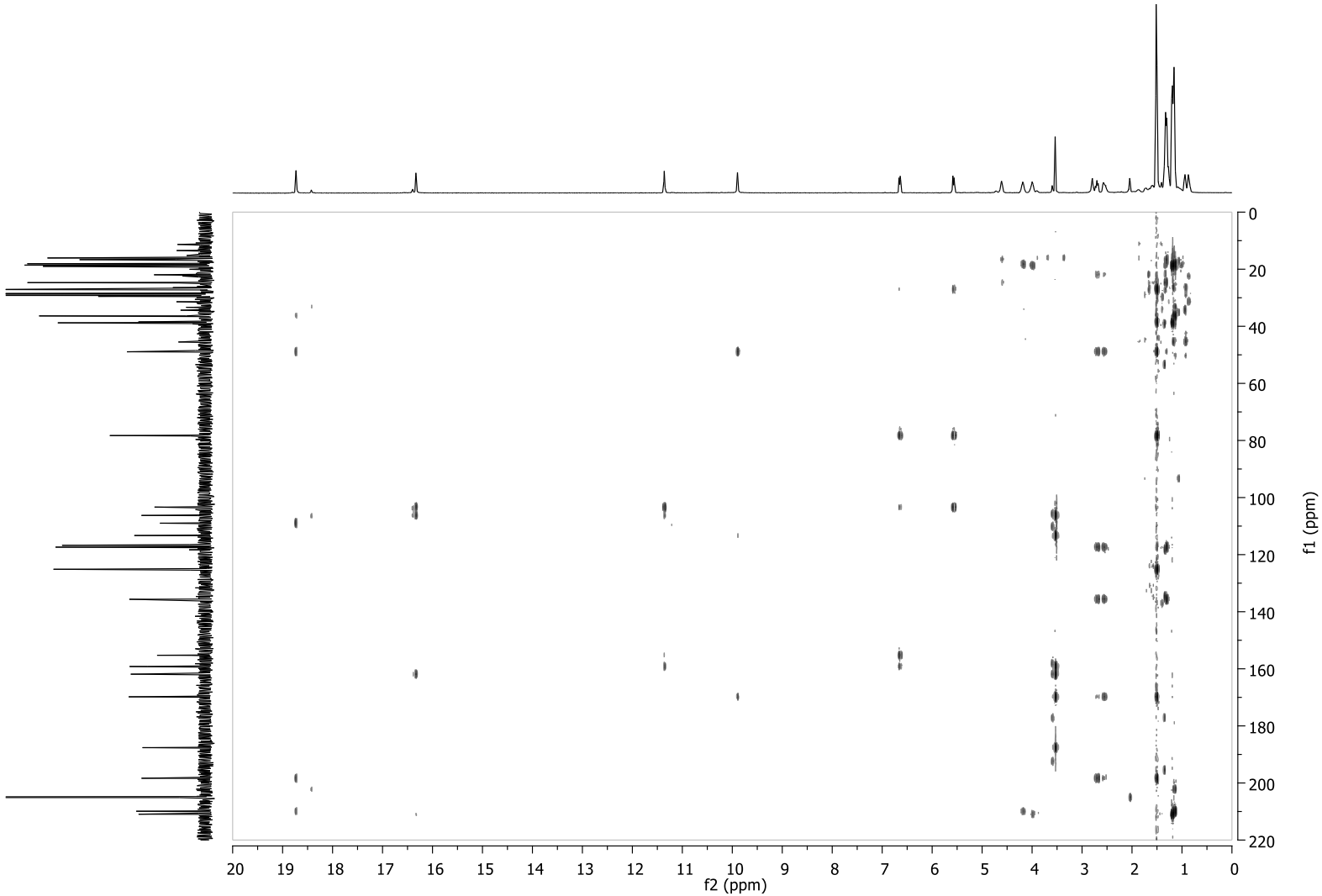

Figura 4.10 Espectro de correlação de acoplamento heteronuclear HMBC de **hiperbrasilol B** em acetona-d_6 a 400/100 MHz

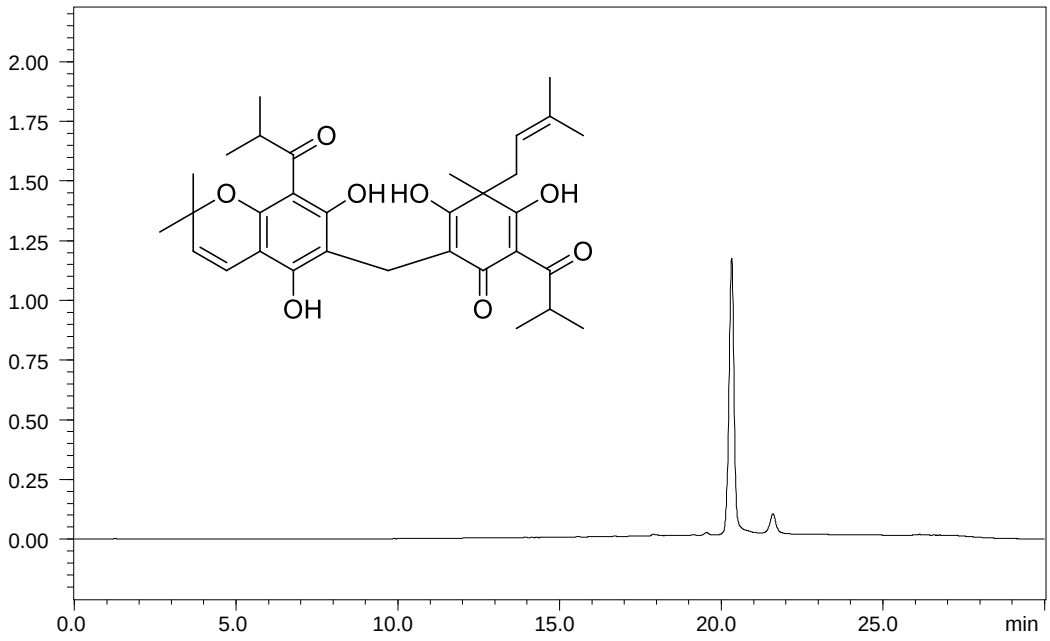

Figure 4.11 Cromatograma por HLPC hiperbrasilol B

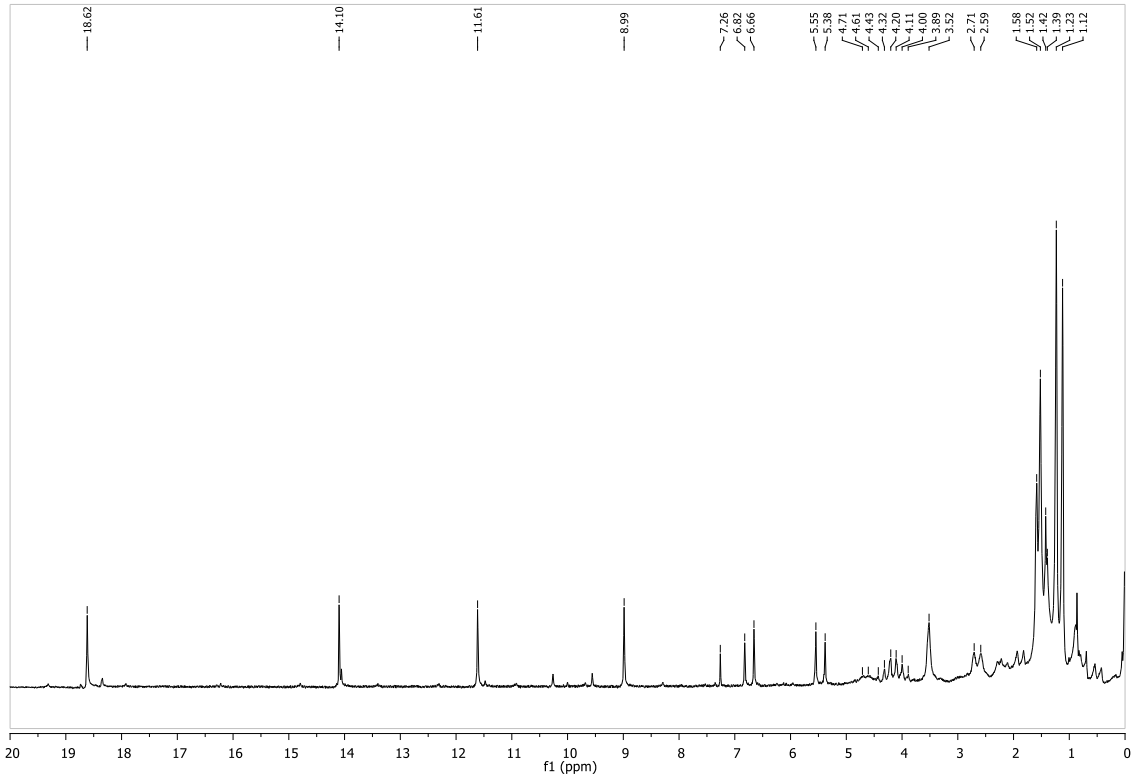

Figura 5.1 Espectro de RMN-^{1}H de **isohiperbrasilol B** em $CDCl_3$ a 60 MHz

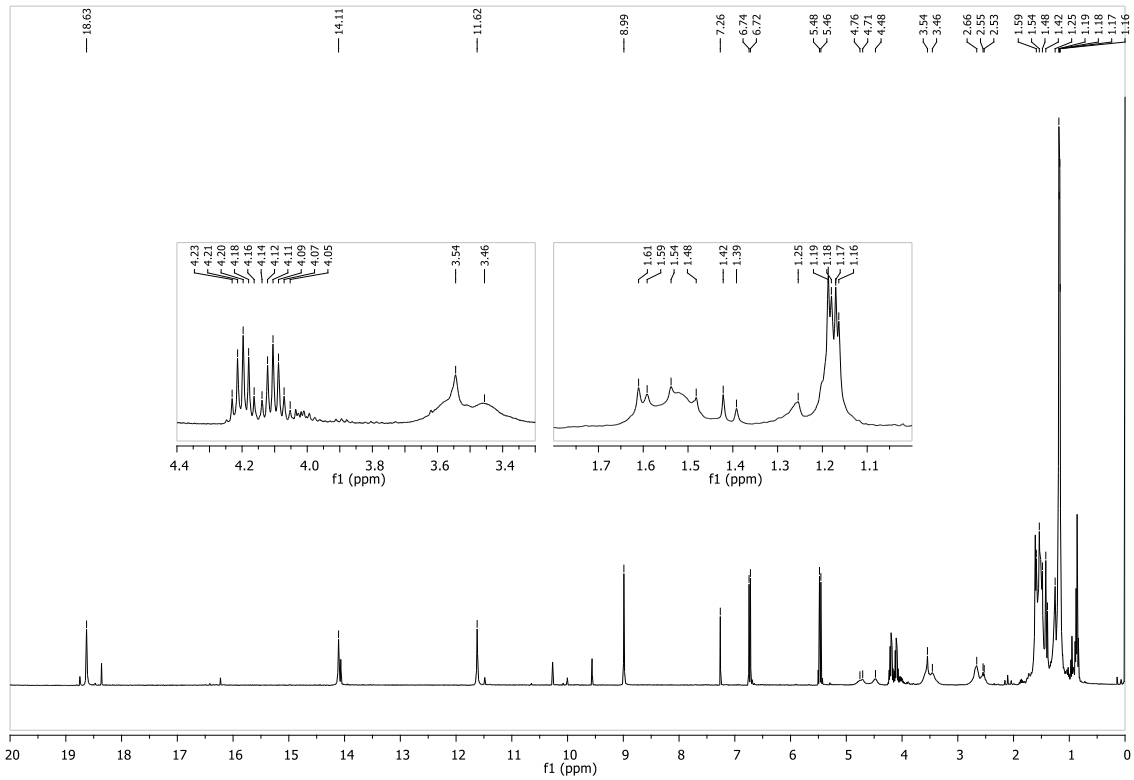

Figura 5.2 Espectro de RMN-^{1}H de **isohiperbrasilol B** em $CDCl_3$ a 400 MHz

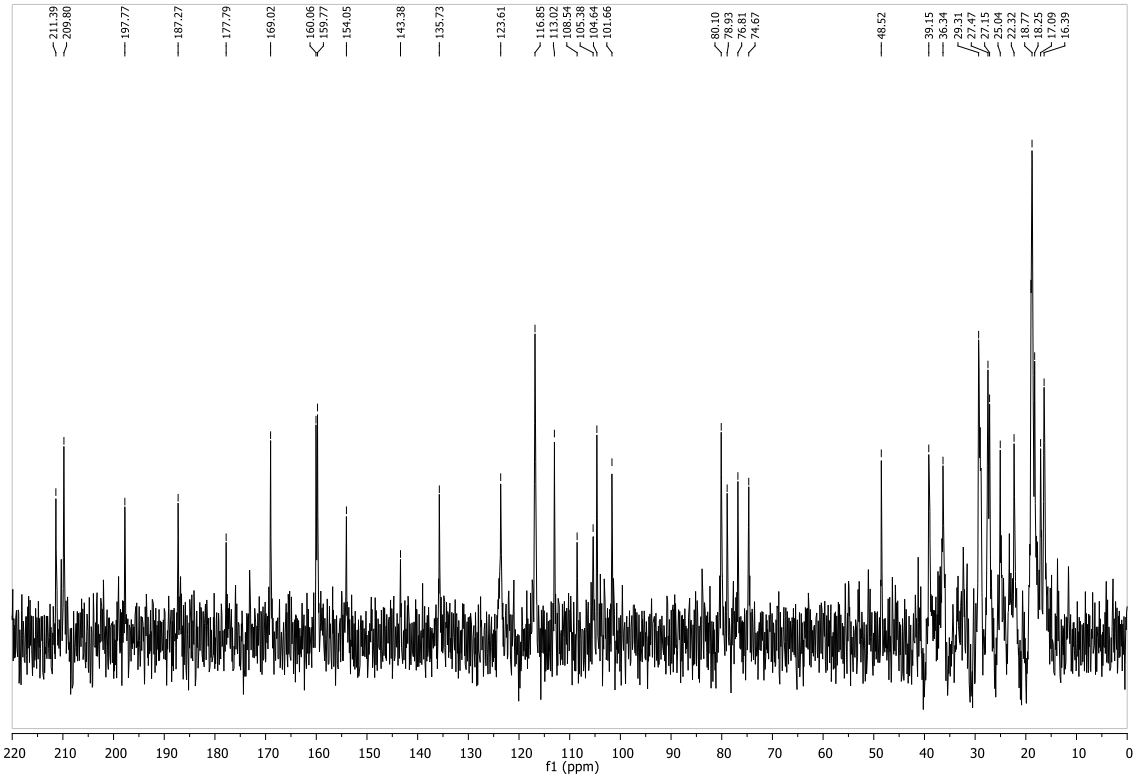

Figura 5.3 Espectro de RMN-^{13}C de **isohiperbrasilol B** em $CDCl_3$ a 15 MHz

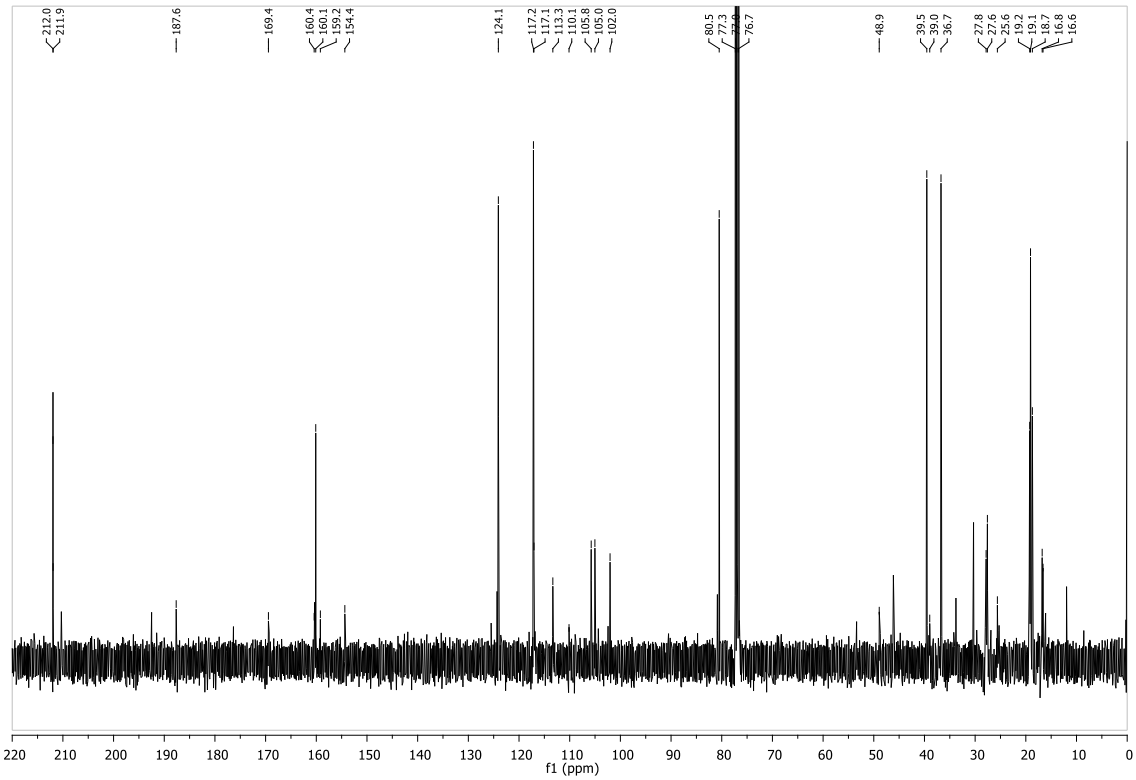

Figura 5.4 Espectro de RMN-^{13}C de **isohiperbrasilol B** em $CDCl_3$ a 100 MHz

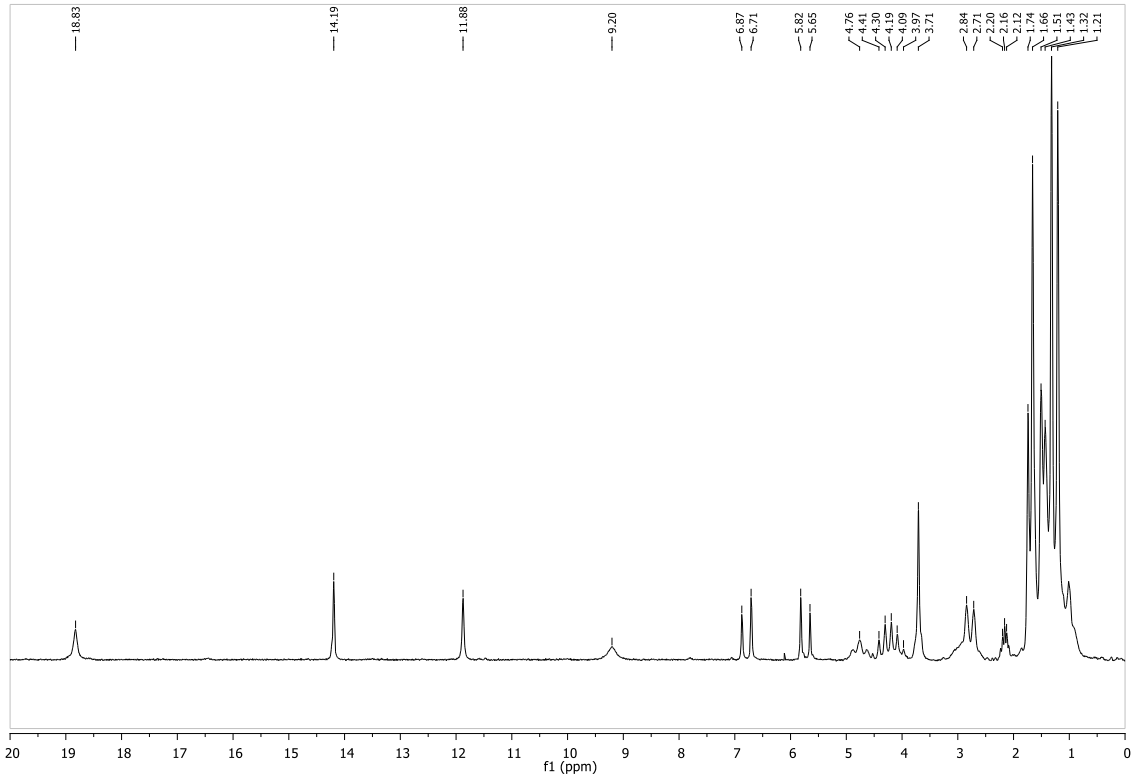

Figura 5.5 Espectro de RMN-^{1}H de **isohipebrasilol B** em acetona-d_6 a 60 MHz

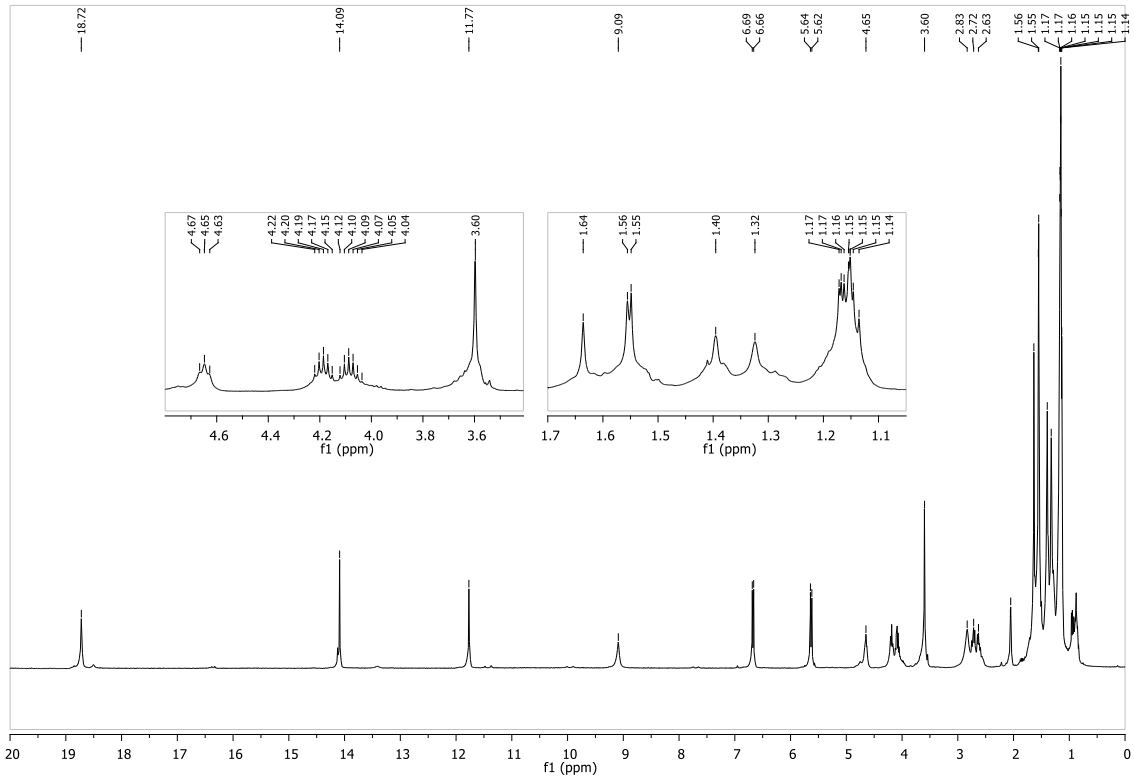

Figura 5.6 Espectro de RMN-^{1}H de **isohipebrasilol B** em acetona-d_6 a 400 MHz

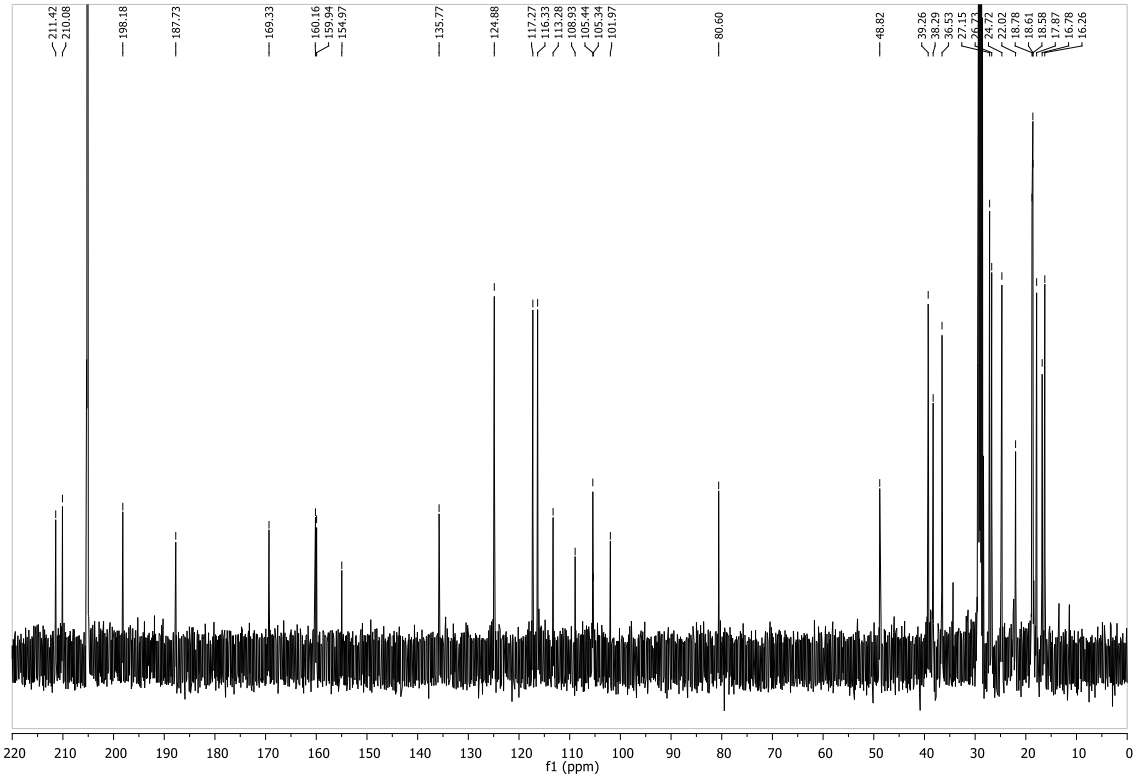

Figura 5.7 Espectro de RMN-^{13}C de **isohipebrasilol B** em acetona-d_6 a 100 MHz

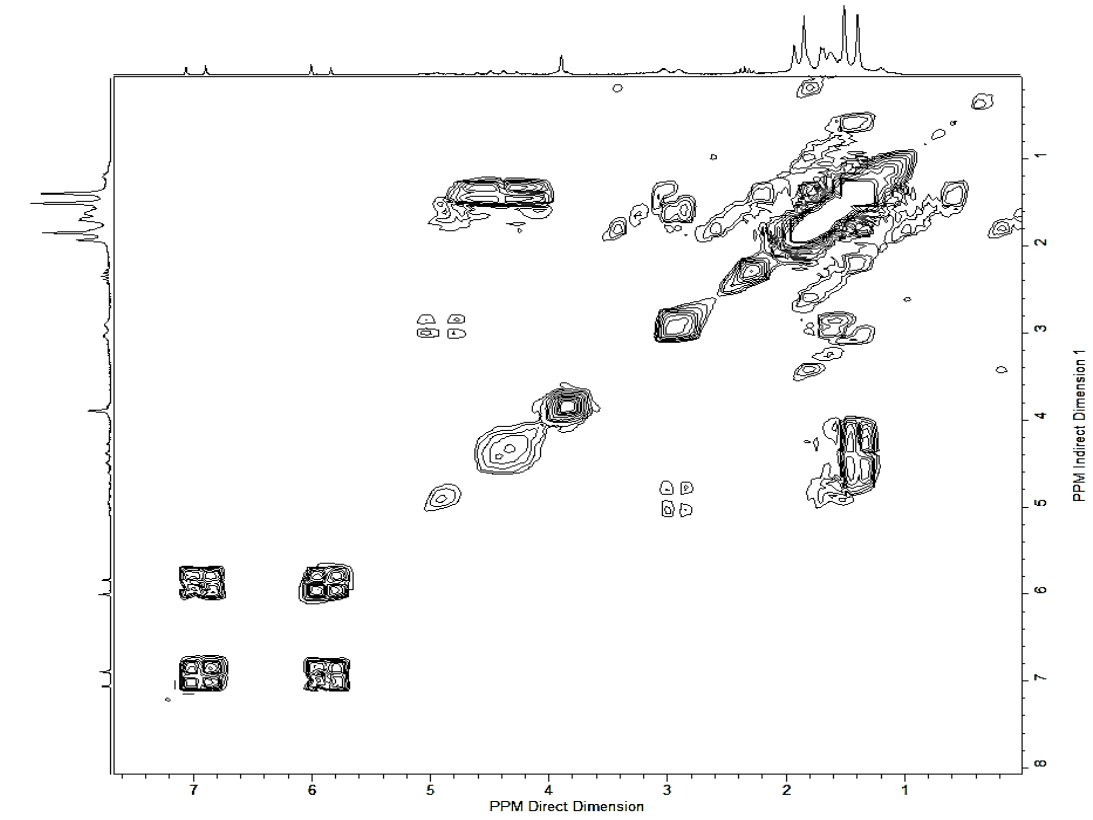

Figura 5.8 Espectro de correlação H-H COSY de **isohiperbrasilol B** em acetona-d_6

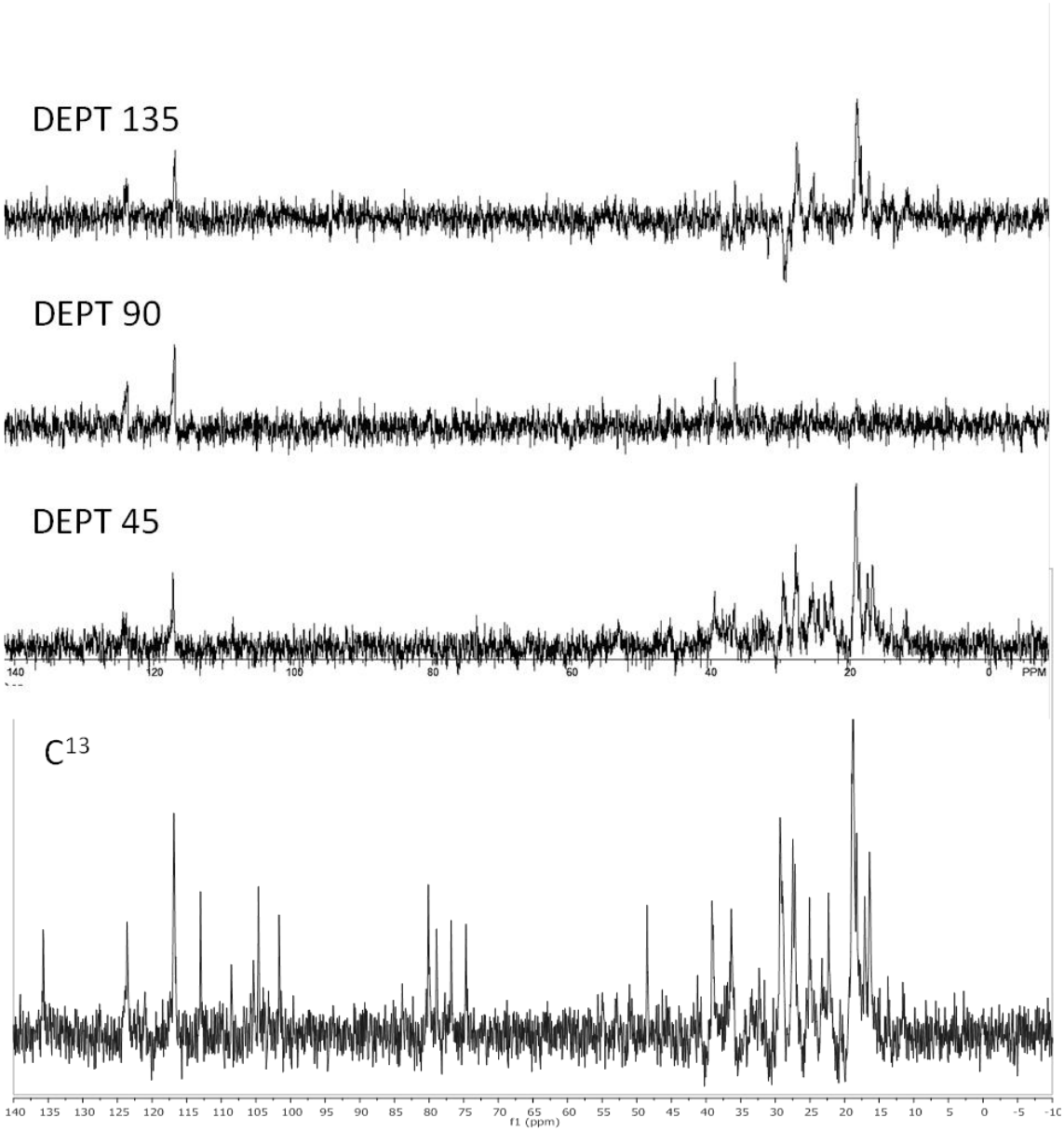

Figura 5.9 Espectro de RMN-^{13}C-DEPT de **isohiperbrasilol B** em $CDCl_3$ a 15 MHz

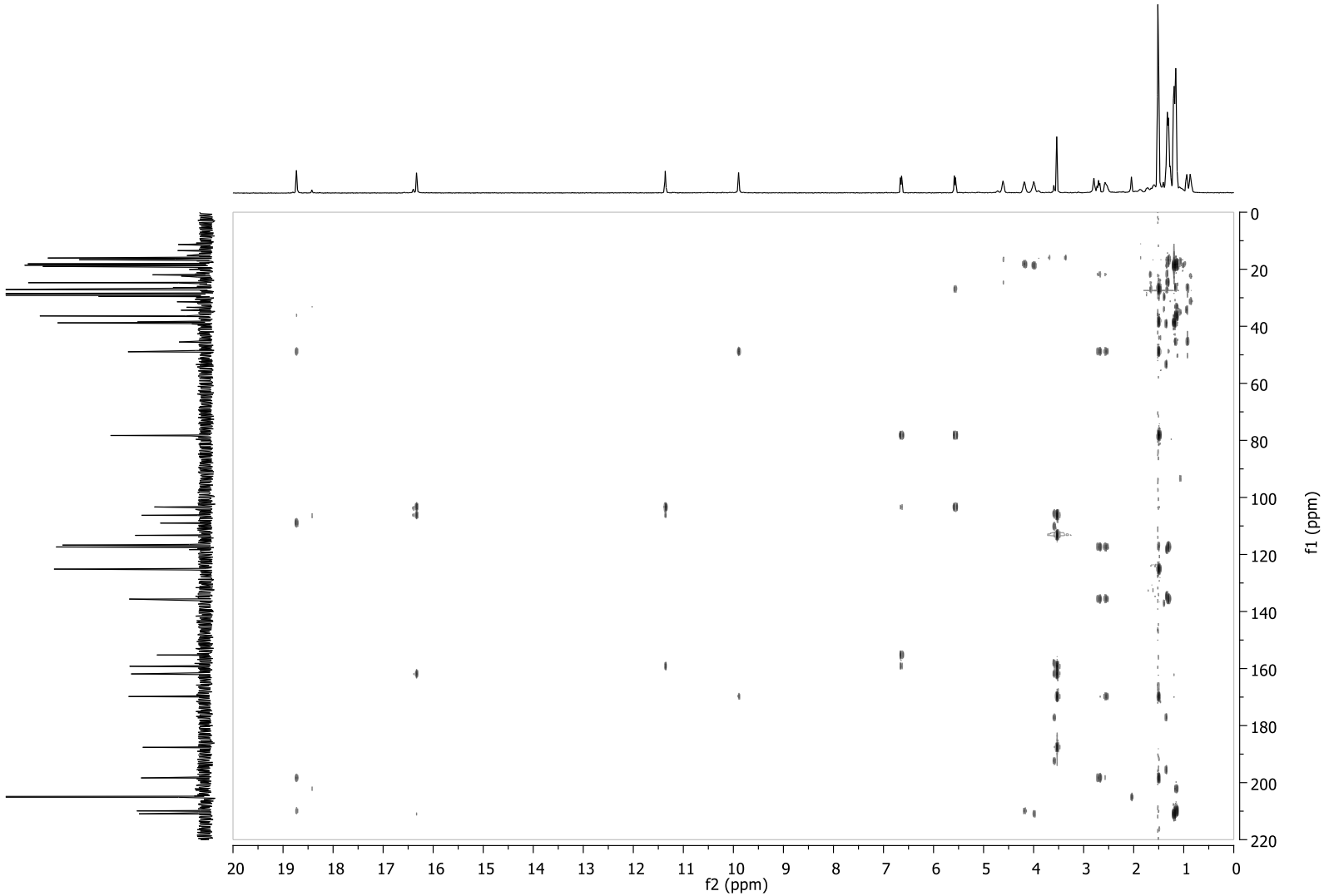

Figura 5.10 Espectro de correlação de acoplamento heteronuclear HMBC de **isohiperbrasilol B** em acetona-d_6 a 400/100 MHz

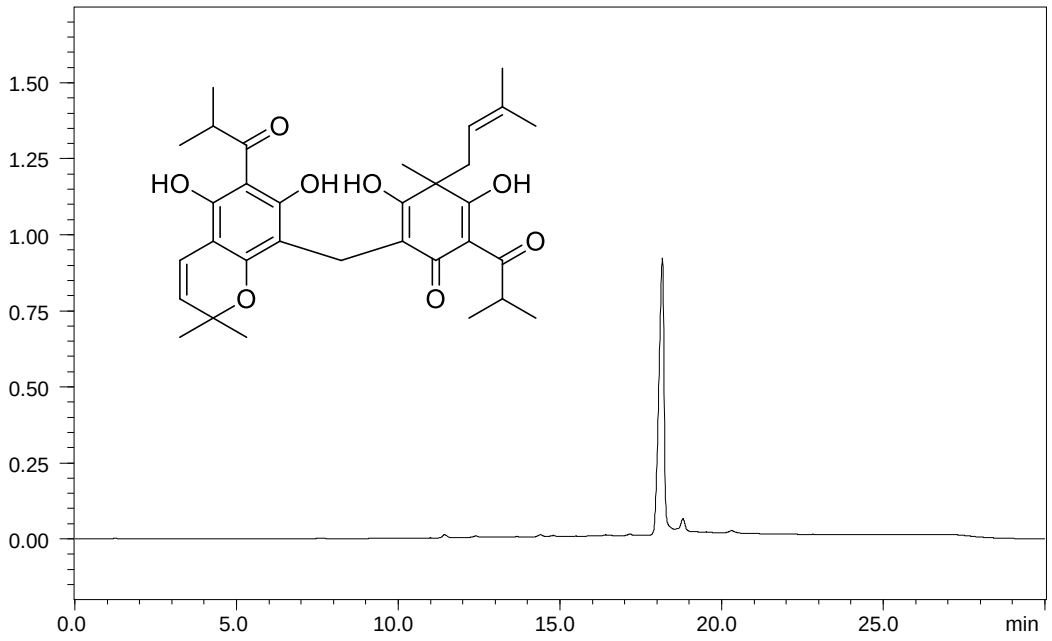

Figure 4.11 Cromatograma por HLPC isohiperbrasilol B

CAPÍTULO II

"Acylphloroglucinol derivatives from *Hypericum andinum* and *H. laricifolium*: antidepressant-like activity of andinin A"

Manuscrito em preparação

Acylphloroglucinol derivatives from *Hypericum andinum* and *H. laricifolium*: antidepressant-like activity of andinin A

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Mental depressive disorders are major public health issues that encourage the search for new therapeutic agents (Ferrari et al. 2013). The herbaceous plant *H. perforatum* (St. John's wort) constitutes one of the most recognized phytotherapeutical preparations for the treatment of mild to moderate depression (Kasper et al. 2010). Flavonoids, naphthodianthrones and acylphloroglucinol derivatives are recognized as mayor contributors to the overall effect observed (Butterweck and Schmidt 2007).

The polycyclic polyisoprenylated acylphloroglucinols hyperforin, adhyperforin and hyperfoliatin are lipophilic constituents of *H. perforatum* and *H. perfoliatum* that display similar pharmacological profiles in animal and biochemical models of antidepressant activity. They are able to potently and nonselectively inhibit the synaptosomal uptake of dopamine, serotonin and noradrenaline and, differently from classical antidepressant drugs, they show no direct binding to any of these monoamine transporters, thus offering a new mechanism of action not related to the current pharmacological treatments for depressive disorders (Chatterjee et al. 1998; Jensen et al. 2001; do Rego et al. 2007).

Differently to the hyperforin-producing (and polyisoprenylated acylphloroglucinol derivatives) *Hypericum* species from sections *Androsaemum*, *Drosocarpium*, *Hypericum*, etc.,

mainly distributed in Africa and Eurasia (Robson 2012; Crockett 2012), the *Hypericum* species from sections *Brathys* and *Trigynobrathys*, mainly distributed in Central and South America, are sources of primarily dimeric structures consisting of an acylfilicinic acid and an acylphloroglucinol moiety linked by a methylene bridge. The occurrence of thirty-one dimeric acylphloroglucinols reported for *Hypericum* species from sections *Brathys* and *Trigynobrathys* was recently reviewed, and the presence of five known dimeric acylphloroglucinols, uliginosin A, uliginosin B, isouliginosin B, hyperbrasilol B and isohyperbrasilol B, in four Peruvian *Hypericum* species was presented for the first time (Ccana-Ccapatinta et al. 2013).

Studies conducted by our group have demonstrated that the dimeric acylphloroglucinol molecular scaffold represents a promising tool for the development of new antidepressants (Socolsky et al. 2012a; Stein et al. 2012). Uliginosin B is the most investigated dimeric acylphloroglucinol and is reported to occur in 19 *Hypericum* species from sections *Brathys* and *Trigynobrathys* (Ccana-Ccapatinta et al. 2013). Uliginosin B present antidepressant-like effect in rodents by an innovative mechanism of action, not completely elucidated. It increases the availability of monoamines in the synaptic cleft, without binding to sites on the monoaminergic neuronal carriers indicating that it acts in a different manner from most antidepressants (Stein et al., 2012). Considering this background and as part of our continuous search for novel structures of dimeric acylphloroglucinol compounds with potential antidepressant-like activity, we have conducted further efforts to isolate and identify additional constituents present in the *n*-hexane extracts of *H. andinum* Gleason and *H. laricifolium* Juss.

Results and Discussion

The air-dried underground plant material of *H. andinum* (roots and stems) and aerial plant material of *H. laricifolium* (stems, leaves and flowers) were ground and extracted by maceration at room temperature with *n*-hexane. The extracts were fractionated, and fractions were further processed as described in the Experimental Section to yield new acylphloroglucinols derivatives andinin A, hyperlaricifolin A, laricifolin A and laricifolin B (**Fig. 1**).

The HRESIMS of **1** showed a pseudomolecular ion peak $[M + H]^+$ at *m/z* 501.2491, consistent with the molecular formula of $C_{28}H_{37}O_8$. The ^1H NMR spectrum of **1** (**Table 1**) exhibited a characteristic signal at very low field (δ_H 18.74) assigned to an enolizable *β*-triketonic system, as previously reported for acylphloroglucinol derivatives carrying an acylfilicinic acid moiety isolated from genus *Hypericum*, *Elaphoglossum* and *Dryopteris* (Ccana Ccapatinta et al. 2013; Socolsky et al 2012a and 2012b). This evidence, in addition to the presence of carbon signals of a carbonyl (C-1, δ_C 187.3), four enol (C-2, δ_C 111.3; C-3, δ_C 171.1; C-5, δ_C 199.0 and C-6, δ_C 107.0), and a dimethyl substituted quaternary carbon (C-4, δ_C 44.1) in the ^{13}C NMR spectrum of **1** confirmed the presence of a dimethyl substituted acylfilicinic acid moiety. The ^1H and ^{13}C NMR spectrum of **1** showed certain anomalies that are best explained by the expected keto-enol tautomerization of the of the acylfilicinic acid moiety in $CDCl_3$ solution. This phenomena complicates spectra interpretation, but in no case prohibited complete assignments. In order to overcome this difficulty and to confirm assignments in $CDCl_3$, further NMR experiments of **1** were recorded in acetone-d_6 (**Table 1**). A characteristic signal for a methylene bridge was observed at δ_H 3.53, providing evidence of the presence of an acylfilicinic acid moiety linked to an aromatic ring by a methylene bridge. In addition, the signals of two acyl residues were clearly detected at δ_C 208.5 and δ_C 207.6 in the ^{13}C NMR spectrum, providing further evidence of the presence of two rings in **1**. The phloroglucinol moiety was inferred as the second ring by the observation of two signals of chelated hydroxyl groups (8'-OH, δ_H 11.55; 10'-OH, δ_H 13.88) and three signals of aromatic oxygen-bearing carbons (C-6', δ_C 160.3; C-8', δ_C 159.7; C-10', δ_C 165.6) in the ^1H and ^{13}C NMR spectra, thus confirming that **1** is a dimeric acylphloroglucinol, consisting of a acylfilicinic acid and a phloroglucinol moiety linked by a methylene bridge. This kind of compounds is frequently isolated from *Hypericum* species from sections *Brathys* and *Trigynobrathys* (Ccana-Ccapatinta et al. 2013). Furthermore, signals of a prenyl side chain were observed (**Table 1**). These 1D-NMR spectroscopic data resemble those of uliginosin A, a dimeric acylphloroglucinol first isolated from *H. uliginosum* (Parker and Johnson 1968). Differently to uliginosin A, compound **1** bears an *O*-prenyl side chain since its methylene signals (δ_H 4.66; δ_C 66.1) displayed more deshielded values compared to the *C*-prenyl side chain of uliginosin A. This side chain is linked to the C-6' of the acylphloroglucinol moiety of **1** as confirmed by HMBC correlations observed in the **Fig. 2**. Hence, compound **1** was identified by spectroscopic analysis as 3,5-dihydroxy-2-isobutyryl-4,4-dimethyl-6(2,4-dihydroxy-3-

isobutyryl-6-((3-methylbut-2-enyl)oxy)benzyl)cyclohexa-2,5-dienone and trivially named andinin A.

The HRESIMS of **2** showed a pseudomolecular ion peak $[M + H]^+$ at *m/z* 623.3566, consistent with the molecular formula of $C_{37}H_{50}O_8$. The ^1H and ^{13}C NMR spectra of **2** in $CDCl_3$ were unresolved but exhibited the characteristic signal of a chelated hydroxyl group at very low field (δ_H 18.57) suggesting the presence of an enolizable *β*-triketonic system. Further experiments of **2** in acetone-d_6 confirmed the presence of a acylfilicinic acid moiety as in as in andinin A (**Table 1**). The signal for a methylene bridge (δ_H 3.54), in addition to those of two acyl residues (δ_C 210.9 and δ_C 211.7), three aromatic oxygen-bearing carbons (C-6', δ_C 162.2; C-8', δ_C 162.7; C-10', δ_C 156.3) and a characteristic quaternary carbon (C-3', δ_C 80.6) signals in the ^1H and ^{13}C NMR spectra of **2** suggested the presence of a acylphloroglucinol moiety with an tetrahydropyran skeletal element in agreement to a 2,2-dimethyl dihydrochromene moiety. Signals corresponding to two prenyl side chain were also observed (**Table 1**). Only one methyl group was found at C-4, in addition the resonance of C-4 (δ_C 49.9) in the ^{13}C NMR spectrum of **2** was shifted ca 5 ppm downfield compared to that of andinin A. Hence, one prenyl side-chain was placed at C-4, together with one methyl group in compliance with a prenylated acylfilicinic acid moiety. Such a deshielding effect on C-4 when a methyl group is replaced by a prenyl chain has been observed in drummondins (Jayasuriya et al. 1989) and hyperbrasilols (Rocha et al. 1995; Rocha et al. 1996). These 1D-NMR spectroscopic data resemble those of hyperbrasilol B, a dimeric acylphloroglucinol first isolated from *H. brasiliense* (Rocha et al. 1996). Distinctively to hyperbrasilol B, compound **2** displayed a set of signals due to and additional prenyl side chain identified as an isopentenylmethyl group that replaced one of the geminal methyl groups of the base 2,2-dimethyl chromene moiety to form an isoprenylmethyl-methyl-chromene system. This moiety has been previously identified in the sarothralens (Ishiguro et al. 1994) and hypercalyxones (Winkelmann et al. 2003) isolated from *H. japonicum* and *H. amblycalyx*, respectively. The fusion of the pyran ring in **2** was inferred by direct comparison of the hydroxyl resonances, in particular 6'-OH (δ_H 11.19) and 8'-OH (δ_H 16.52), with those observed in the regioisomers hyperbrasilol B and isohyperbrasilol B. The reliability of these hydroxyl signals for the determination of the cyclisation pattern of oxygenated chromenes was confirmed by SINEPT experiments on drummondin C and isodrummondin C (Jayasuriya et al. 1994). These 1D observations were confirmed by HSQC and HMBC experiments. Therefore, compound **2** was identified by

spectroscopic analysis as 2-((5,7-dihydroxy-8-isobutyryl-2-methyl-2-(4-methylpent-3-enyl)chroman-6-yl)methyl)-3,5-dihydroxy-6-isobutyryl-4-methyl-4-(3-methylbut-2-enyl)cyclohexa-2,5-dienone and trivially named hyperlaricifolin A. This compound has two stereogenic centers at C-4 and C-3' whose absolute configuration could not be determined.

Duplicated and/or overlapping ^{1}H and ^{13}C NMR patterns, in a ratio of approximately 1:1 (derived from the ^{1}H and ^{13}C NMR signal intensities), and the presence of two pseudomolecular ion peaks at *m/z* 355.1946 $[M + Na]^{+}$ and 369.2106 $[M + Na]^{+}$ in the HRESIMS, indicated that **3**/**4** was obtained as a mixture of closely related compounds, which were later shown to be M+14 homologue forms consistent with the molecular formula of $C_{20}H_{28}O_4$ and $C_{21}H_{30}O_4$. Both sets of signals in the ^{13}C NMR spectrum showed almost identical chemical shifts (eight quaternary carbons, two methylenes, four methines, and six methyl groups in **3**, while **4** displayed and additional methylene group). The presence of duplicated signals for a chelated hydroxyl (δ_H 14.45, δ_H 14.10), an acyl residue (C-12, δ_C 210.5; C-12, δ_C 210.6), and a carbocyclic ring with three oxygen bearing carbon units (C-6, δ_C 161.3; C-8, δ_C 164.7; C-10, δ_C 160.5 for **3** and C-6, δ_C 161.3; C-8, δ_C 164.7; C-10, δ_C 160.5 for **4**) in the ^{1}H and ^{13}C NMR spectrum of **3**/**4** suggested the presence of a monomeric acylphloroglucinol moiety. Two additional set of signals consistent with two prenyl (**Table 1**) side chains in **3**/**4** was also observed as a common feature. However, one prenyl side chain is linked to the acylphloroglucinol moiety by an ether linkage constituting an *O*-prenyl while the other constitutes a *C*-prenyl side chain. This was inferred since the methylene signals of the *O*-prenyl (C-5, δ_C 65.4, δ_H 4.51 for **3**) displayed more deshielded values compared to the *C*-prenyl side chain (C-1', δ_C 21.6; δ_H 3.37 for **3**). The principal difference between **3** and **4** is the presence of an additional methylene group in **4**. This additional methylene signal was located at the acyl side chain. This was consistent with the presence of an *iso*-butyryl (C-13, δ_C 39.4, δ_H 3.80 *sep* *J*=6.8) and a 2-methylbutyryl (C-13, δ_C 46.1, δ_H 3.66 *sex* *J*=6.8) functionality as inferred by its characteristic multiplicities in the ^{1}H NMR spectrum of **3**/**4** and further confirmed by COSY, HSQC and HMBC experiments. The *orto* and *meta* placement of the *O*-prenyl and *C*-prenyl side chains, respectively, with respect to the acyl residue was determined and confirmed by HMBC correlations observed in the **Fig. 3**. Hence, compounds **3/4** were identified by spectroscopic analysis as 1-(2,4-dihydroxy-3-(3-methylbut-2-enyl)-6-((3-methylbut-2-enyl)oxy)phenyl)-2-methylpropan-1-one (**3**) and 1-(2,4-dihydroxy-3-(3-

methylbut-2-enyl)-6-((3-methylbut-2-enyl)oxy)phenyl)-2-methylbutan-1-one (**4**) and trivially named laricifolin A (**3**) and laricifolin B (**4**).

The results displayed in **Fig. 4** showed that the administration of andinin A (**1**) produced a significant reduction in the immobility time of mice submitted to the forced-swimming test (FST) relative to the vehicle-treated group (ANOVA: $F_{(5,60)}= 16.032$, $p<0.001$). This effect was observed at doses of 3, 10 and 30 mg/kg, p.o. and presented a magnitude of effect comparable to imipramine 20 mg/kg, p.o. (reference drug control). In the FST the immobility postures are observed and assumed to be a depressive-like parameter that is considered as an expression of "behavioral despair"; however drugs that alter motor behavior can induce false results. Andinin A (**1**) did not altered the locomotor activity at the highest dose (30 mg/kg, p.o.) in the open field test (*t*-Student test: $p=0.249$), revealing that the anti-immobility effect observed in the FST was not related to a nonspecific motor stimulation (data not shown).

Dimeric acylphloroglucinols crassipin A and uliginosin B have been reported to reduce the immobility time in the mice FST at doses of 15 mg/kg, p.o. and 10 mg/kg, p.o., respectively (Socolsky et al. 2012a; Stein et al. 2012). In the same way as the polyisoprenylated acylphloroglucinols hyperforin, adhyperforin and hyperfoliatin, the dimeric acylphloroglucinol uliginosin B was able to inhibit non selectively the synaptosomal uptake of dopamine, serotonin and noradrenaline, with IC_{50} values in the nanomolar range, without binding to any of these monoamine transporters (Stein et al. 2012). These acylphloroglucinol derivatives constitute not only new alternatives for treatment of depression but also introduce a new mechanism of action, which could inspire the design of new antidepressants (Stein et al. 2012; Richard 2014). The present data allow to suggest that andinin A (**1**) deserves further studies in other animal models predictive of antidepressant activity, as well as, studies to elucidate the mechanism of action, since andinin A (**1**) presented significant effect in doses (in molar concentration) 3 to 4 times lesser than the others dimeric acylphloroglucinol previously evaluated (Socolsky et al. 2012a; Stein et al., 2012)..

These data set observations added to previous results allow us to suggest that *Hypericum* species from sections *Brathys* and *Trigynobrathys* are sources of dimeric acylphloroglucinol derivatives with potential antidepressant-like activity, which can represent new chemical scaffolds for the design of antidepressants with new mechanism of action.

Experimental Section

General Experimental Procedures. ^{1}H, ^{13}C, and 2D NMR spectra were measured at 25 ºC on a Varian MR400 spectrometer (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C). Spectra were recorded in $CDCl_3$ (99.8%, Acros Organics, New Jersey, USA), with tetramethylsilane (TMS) as internal standard, and acetone-d_6 (99.9%, Sigma Aldrich, St. Louis, MO, USA) referenced against residual non deuterated solvent (acetone-d_6: δ_H 2.05/δ_C 29.8). . The 1D and 2D NMR (COSY, HMBC and HSQC) spectra were obtained by using the standard pulse sequences from Varian user library. Spectra of isolated compounds are provided as Supporting Information. HRESIMS were acquired in positive-ion mode on a Q-TOF Premier spectrometer equipped with a nanospray ion source (Waters, Milford, MA, USA). Reagent grade Acetone (Acet), dichloromethane (DCM), ethyl acetate (EtOAc), *n*-hexane (Hex) (F. Maia, São Paulo, Brazil), formic acid (Vetec, Rio de Janeiro, Brazil), and HPLC grade acetonitrile and methanol (Merck, Darmstadt, Germany) were regularly used in the extraction and isolation procedures. Dry column vacuum chromatography (DVCC) was carried out over silica gel H (10 – 40 µm, Merck, Darmstadt, Germany). Column chromatography (CC) was carried out over silica gel 60 (70−230 mesh, Merck, Darmstadt, Germany). The extracts and fractions were monitored by TLC on precoated silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) as a stationary phase using several different mobile phases composed of: Hex/DCM (50:50 v/v), Hex/EtOAc (90:10 v/v), Hex/EtOEt (95:5 v/v), Hex/Acet (95:5 v/v), Hex/EtOAc (95:5 v/v), Hex/EtOEt (97.5:2.5 v/v) and Hex/Acet (97.5:2.5 v/v), these three later acidified with 0.25 % formic acid. After elution, acylphloroglucinols were detected by fluorescence quenching at 254 nm, by dark-blue-colored spots at 356 nm, and by a brown (**1**/**2**), orange (**3**) and yellow (**4**) spot colors after spraying with anisaldehyde/sulfuric acid reagent. Centrifugal planar chromatography (CPC) was carried out on silica gel 60 G F_{254} (1 mm plates) using a Chromatotron^{TM} instrument (model 7924 T, Harrison Research, San Bruno, CA, USA).

Plant material. The underground parts of *Hypericum andinum* Gleason (Hypericaceae) were collected in Amparaes, Calca Province, Cusco, while aerial parts of *H. laricifolium* Juss were collected in Cumbemayo, Cajamarca Province, Cajamarca, under consent of Dirección General Forestal y de Fauna Silvestre of the Republic of Peru (0147-2010-AG-DGFFS-

DGEFFS). The plants were identified by Prof. Washington H. Galiano Sánchez, Academic Department of Biology, University of San Antonio Abad del Cusco, Peru. Voucher specimens were deposited in the Herbarium of the Federal University of Rio Grande do Sul (ICN), Brazil and in the Herbarium Vargas (CUZ) of the National University of San Antonio Abad del Cusco, Peru.

Extraction and isolation. The air-dried and powdered vegetal material (*H. andinum*, 3.50 kg; *H. laricifolium*, 0.85 kg) was successively extracted by maceration with *n*-hexane over 72 h (5 times, plant solvent ratio 1:5). The extracts were joined and evaporated to dryness under reduced pressure and then treated with cold acetone to obtain insoluble fatty residues and acetone-soluble fractions (ASF) that yielded dark-greenish viscous oil residues after solvent evaporation (62.5, and 12.2 g for *H. andinum* and *H. laricifolium*, respectively).

The ASF of *H. andinum* (62.5 g) was subjected to DVCC on silica gel H using Hex/EtOAc gradient as mobile phase. A first bright yellow broad band was eluted with Hex/EtOEt (100:0–90:10) while a second greenish band rest on application point and eluted only after increasing mobile face polarity (90:10–80:20) that were discharged since no acylphloroglucinol profile was seen on TLC. This initial fraction (40.2 g) was resubmitted to DVCC (100:0–90:10) to obtain an initial bright yellow fraction (100:0–98:2) rich in uliginosin B and two further reddish fractions (A, 97:3–95:5 and B, 95:5–90:10). Fraction A (4.2 g) was submitted to CC using Hex/EtOAc (100:0–90:10) gradient as mobile phase to afford eight subfractions (SbFr. 1–8). SbFr. 3 (980 mg) was subjected to repeated CPC on 1 mm silica gel plates using gradients of Hex/EtOAc (100:0–90:10), Hex/EtOEt (100:0–95:5), Hex/Acet (100:0–95:5), with/without 0.25% formic acid, as mobile phases to afford 23 mg of **1,** uliginosin A and isouliginosin B.

The ASF of *H. laricifolium* (12.2 g) was subjected to silica gel CC using Hex/DCM gradient as mobile phase to afford twelve bright yellow-reddish fractions (100:0–90:10; Fr. 1–12). Fr. 2–3 (450 mg) were subjected to CC using Hex/EtOAc gradient as mobile phase to afford four subtractions (SbFr. 1–4). SbFr. 2 and SbFr. 3 afforded hyperbrasilol B and isohyperbrasilol B, respectively. SbFr.1 (60 mg) was subjected to CPC on a 1 mm silica gel plate using Hex/EtOAc gradient as mobile phase to afford 7 mg of **2**. SbFr. 4 (32 mg) was subjected to CC using Hex/EtOAc gradient as mobile phase to afford isohyperbrasilol B and 10 mg of **3**/**4**.

On TLC **3**/**4** appeared as a single spot under several mobile face systems described in the Experimental Section. Further attempts to separate 1/2 by CPC were fruitless. Fr. 6-7 afforded uliginosin B and isouliginosin B.

Andinin A (**1**) 3,5-dihydroxy-2-isobutyryl-4,4-dimethyl-6(2,4-dihydroxy-3-isobutyryl-6-((3-methylbut-2-enyl)oxy)benzyl)cyclohexa-2,5-dienone: yellow oil ^{1}H NMR and ^{13}C NMR see **Table 1**; ESIMS *m/z* 501 $[M + H]^+$ (6), 445 (17), 433 (63) 375 (27), 277 (13), 265 (24), 237 (70), 225 (25), 209 (51), 197 (100), 69 (8); HRESIMS *m/z* 501.2491 $[M + H]^+$ (Calcd for $C_{28}H_{37}O_8$, 501.2488);

Hyperlaricifolin A (**2**) 2-((5,7-dihydroxy-8-isobutyryl-2-methyl-2-(4-methylpent-3-enyl)chroman-6-yl)methyl)-3,5-dihydroxy-6-isobutyryl-4-methyl-4-(3-methylbut-2-enyl)cyclohexa-2,5-dienone: yellow oil; ^{1}H NMR and ^{13}C NMR see **Table 1**; ESIMS *m/z* 623$[M + H]^+$ (15), 499 (5), 345 (100), 333 (9), 221 (4); HRESIMS *m/z* 623.3566 [M+H]+ (Calcd for $C_{37}H_{51}O_8$, 623.3584)

Laricifolin A/B (**3/4**) 1-(2,4-dihydroxy-3-(3-methylbut-2-enyl)-6-((3-methylbut-2-enyl)oxy)phenyl)-2-methylpropan-1-one / 1-(2,4-dihydroxy-3-(3-methylbut-2-enyl)-6-((3-methylbut-2-enyl)oxy)phenyl)-2-methylbutan-1-one: white amorphous powder; ^{1}H NMR and ^{13}C NMR see **Table 2**; ESIMS (**3**) *m/z* 355 $[M + Na]^+$ (3), 333 $[M + H]^+$ (11), 277 (51), 265 (64), 209 (100), 191 (14), 151 (8), 69 (1), (**4**) 369 $[M + Na]^+$ (3), 347 $[M + H]^+$ (13), 291 (56), 279 (67), 223 (100), 207 (88), 205 (22), 151 (9), 69 (1); HRESIMS (**3**) *m/z* 355.1946 $[M + Na]^+$ (calcd for $C_{20}H_{28}O_4Na$ 355.1964), (**4**) *m/z* 369.2106 $[M + Na]^+$ (calcd for $C_{21}H_{30}O_4Na$ 369.2120);

Behavioral experiments

Animals. Adult male CF1 mice (25–35 g) were purchased from the Fundação Estadual de Produção e Pesquisa em Saúde (Brazil) colony. The animals were housed 6 mice per plastic cages (L: 28 cm, W: 17 cm, H: 13 cm) under a 12 hours light/dark cycle (lights on at 7:00 hour) at constant temperature (23 ± 1ºC) with free access to standard certified rodent diet (Nuvilab CR-1®) and tap water. All experiments were approved by a local Ethics Committee of Animal Use (UFRGS, number 23825/2012) and were in compliance with Brazilian law

(2008; 2013a; 2013b) and Council for International Organization of Medical Sciences International guiding principles for biomedical research involving animals (Bankowski and Howard-Jones 1985).

Treatments. Imipramine (Henrifarma Produtos Químicos e Farmacêuticos LTDA®, São Paulo, Brazil) was dissolved in saline (0.9% NaCl) and andinin A was suspended in saline with 2% polysorbate 80 (Merck, Darmstadt, Germany). All solutions were prepared freshly on test day and administered *per os* (p.o.) at 1 mL/ 100 g body weight. The doses of andinin A (**1**) used in the forced swimming test were 1, 3, 10 or 30 mg/kg, and only the highest dose (30 mg/kg) was evaluated in open field test. Vehicle (saline plus 2% polysorbate 80) and imipramine 20 mg/kg were used as control. The drug concentrations in saline solution were: imipramine – 2 mg/mL; andinin A (**1**) was suspended in vehicle at different concentrations depending on the dose required: 0.1, 0.3, 1 or 3 mg/mL.

Forced-Swimming Test. The FST was conducted using the method described by Porsolt et al. 1977 with minor modifications previously standardized and validated (Stein et al. 2012). Briefly, mice were treated with andinin A (**1**), imipramine or vehicle and forced individually to swim in a cylinder pool (10 cm diameter, 13 cm hight, 22 ±1°C) sixty minutes later; the total time of immobility during a 6 min test was scored and determined by a blinded observer. Each mouse was considered to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water.

Open-Field Test. In order to rule out any unspecific locomotor effect, mice were treated with andinin A (**1**) or vehicle and after sixty minutes were placed individually in an acrylic box (40 × 30 × 30 cm), with the floor divided into 24 equal squares. The number of crossings in the squares with the four paws was measured during a period of 6 min by a blinded observer.

Statistical Analysis. The data were evaluated using *t*-Student test or one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test depending on the experimental design. All results were expressed in mean ± S.E.M. The analyses were performed using Sigma Stat 3.2 software (Jandel Scientific Corporation®). Differences were considered statistically significant at $p<0.05$.

Associated Content

Supporting information. ESIMS and NMR spectra of compounds **1**- **4**.

Acknowledgments

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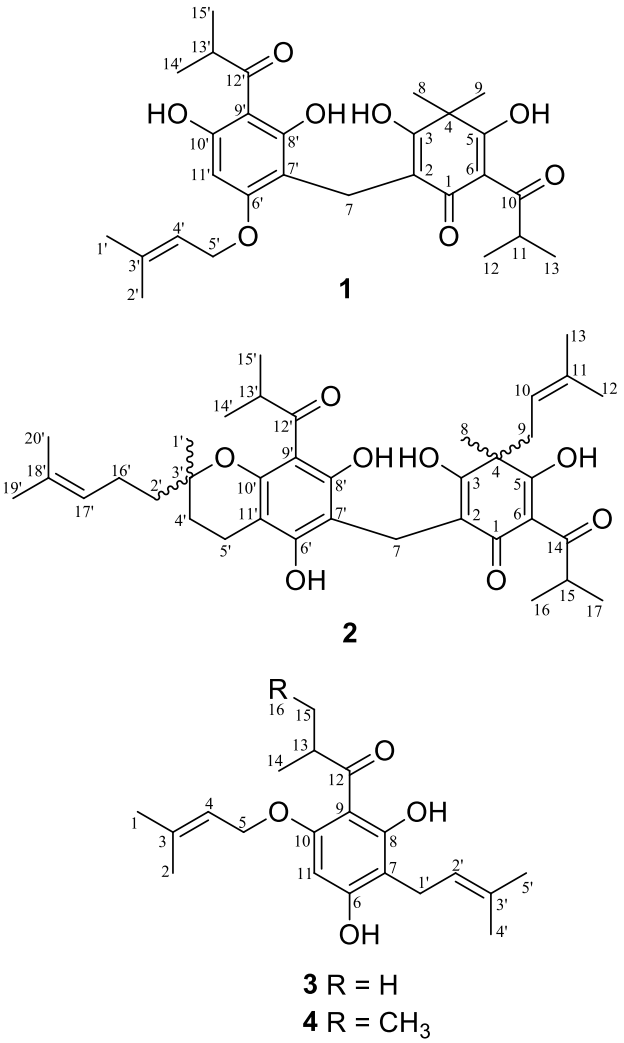

Fig. 1 Structure of compounds **1**-**4**

Table 1 ^{1}H and ^{13}C NMR data of compounds **1** ($CDCl_3$ and acetone-d_6) and **2** (Acetone-d_6)

	1		**1**		**2**	
	δ_C, type	**δ_H, (*J* in Hz)**	**δ_C, type**	**δ_H, (*J* in Hz)**	**δ_C, type**	**δ_H, (*J* in Hz)**
1	187.3, C	-	187.8, C	-	188.6, C	-
2	111.3, C	-	111.8, C	-	114.5, C	-
3	171.1, C	-	171.6, C	-	170.7, C	-
4	44.1, C	-	44.5, C	-	49.9, C	-
5	199.0, C	-	199.7, C	-	199.3, C	-
6	107.0, C	-	107.6, C	-	110.0, C	-
7	17.1, CH_2	3.53 *br s*	17.3, CH_2	3.54 *s*	17.0, CH_2	3.54 *br s*
8	24.8, CH_3 unres	1.49 *br s*	24.7, CH_3	1.50 *s*	22.9, CH_3	1.52 s
9	24.5, CH_3 unres	1.37 *br s*	24.7, CH_3	1.31 *s*	39.4, CH_2	2.8-2.5 *m*
10	210.9, C	-	211.6, C	-	118.3, CH	4.6 *br d*
11	36.7, CH	4.13 *m J*=6.8	37.1, CH	4.10 *sep J*=6.8	136.6, C	
12	18.9, CH_3	1.18 *d J*=6.3	19.2, CH_3	1.16 *d J*=6.8	25.7, CH_3	1.30 *br s*
13	18.9, CH_3	1.18 *d J*=6.3	18.2, CH_3	1.16 *d J*=6.8	17.6, CH_3	1.28 *br s*
14	-	-	-	-	211.7, C	-
15	-	-	-	-	39.7, CH	4.00 *sep J*=6.7
16	-	-	-	-	20.2, CH_3	1.16 *m*
17	-	-	-	-	19.6, CH_3	1.17 *m*
1'	25.9, CH_3	1.86 *s*	25.7, CH_3	1.86 *s*	27.7, CH_3	1.52 *s*
2'	18.2, CH_3	1.79 *s*	17.9, CH_3	1.85 *s*	41.5, CH_2	1.77 *m*
3'	142.4, C	-	142.2, C	-	80.6, C	-
4'	116.8, CH	5.54 *pseudo t J*= 7.0	117.9, CH	5.61 *m*	23.4, CH_2	2.79 *m*; 2.18 *m*
5'	66.1, CH_2	4.66 *br s*[a]	66.7, CH_2	4.79 *d J*=7.2	30.0, CH_2[b] buried	2.32 *unres*; 1.90 *unres*
6'	160.3, C	-	160.2, C	-	162.2, C	-
7'	105.2, C	-	106.1, C	-	106.3, C	-
8'	159.7, C	-	159.9, C	-	162.7, C	-
9'	106.1, C	-	106.0, C	-	104.2, C	-
10'	165.6, C	-	166.2, C	-	156.3, C	-
11'	92.8, CH	6.10 *s*	93.6, CH	6.18 *s*	103.4, C	-
12'	211.7, C	-	211.7, C	-	210.9, C	-
13'	39.4, CH	4.22 *m J*=6.8	39.7, CH	4.21 *sep J*=6.8	37.3, CH	4.18 *sep J*=6.8
14'	18.9, CH_3	1.18 *d J*=6.3	19.2, CH_3	1.16 *d J*=6.8	19.5, CH_3	1.18 *m*
15'	18.9, CH_3	1.18 *d J*=6.3	18.2, CH_3	1.16 *d J*=6.8	19.0, CH_3	1.19 *m*
16'	-	-	-	-	20.9, CH_2	1.29 *br s*
17'	-	-	-	-	123.4, CH	5.23 *pseudo t J*=7.2
18'	-	-	-	-	133.6, C	-
19'	-	-	-	-	26.0, CH_3	1.72 *s*
20'	-	-	-	-	18.0, CH_3	1.61 *s*
3-OH	-	8.99 *s*	-	9.14 *s*	-	10.07 *s*
5-OH	-	18.74 *s*	-	18.81 *br s*	-	18.72 *s*
6'-OH	-	-	-	-	-	11.19 *s*
8'-OH	-	11.55 *s*	-	11.72 *s*	-	16.52 *s*
10'-OH	-	13.88 *s*	-	13.65 *s*	-	-

[a]4.65 *d J*=7.2, ^{1}H NMR spectrum recorded at 60 MHz.
[b]Tentatively assigned.

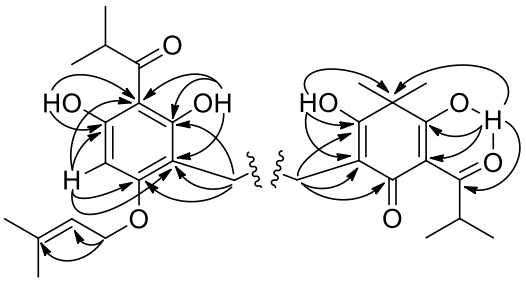

Fig. 2 Key HMBC correlations of andinin A (**1**)

Table 2 ^{1}H and ^{13}C NMR data of compounds **3**/**4** ($CDCl_3$)

	3		**4**	
	δ_C, type	**δ_H, (*J* in Hz)**	**δ_C, type**	**δ_H, (*J* in Hz)**
1	25.7, CH_3	1.76 *s*	25.7, CH_3	1.76 *s*
2	18.2, CH_3	1.86 *s*	18.1, CH_3	1.86 *s*
3	138.7, C	-	138.5, C	-
4	118.7, CH	5.48 *br s*	118.6, CH	5.48 *br s*
5	65.4, CH_2	4.51 *br s*	65.3, CH_2	4.51 *br s*
6	161.3, C	-	161.3, C	-
7	106.0, C	-	106.0, C	-
8	164.7, C	-	164.7, C	-
9	105.5, C	-	105.0, C	-
10	160.5, C	-	160.4, C	-
11	91.5, CH	5.90 *s*	91.5, CH	5.90 *s*
12	210.6, C	-	210.5, C	-
13	39.4, CH	3.80 *sep J*=6.8	46.1, CH	3.66 *sex J*=6.8
14	19.4, CH_3	1.14 *d J*=6.8	16.5, CH_3	1.11 *d J*=7.2
15	19.4, CH_3	1.14 *d J*=6.8	26.9, CH_2	1.35 *m J*=7.0
16	-	-	11.8, CH_3	0.88 *t J* 7.2
1'	21.6, CH_2	3.37 *d J*=7.2	21.6, CH_2	3.37 *d J*=7.2
2'	121.9, CH	5.27 *pseudo t J*=7.0	121.9, CH	5.27 *pseudo t J*=7.0
3'	135.6, C	-	135.6, C	-
4'	25.8, CH_3	1.73 *s*	25.8, CH_3	1.73 *s*
5'	17.9, CH_3	1.80 *s*	17.9, CH_3	1.80 *s*
6-OH	-	6.15 *br s*	-	6.15 *br s*
8-OH	-	14.45 *s*	-	14.40 *s*

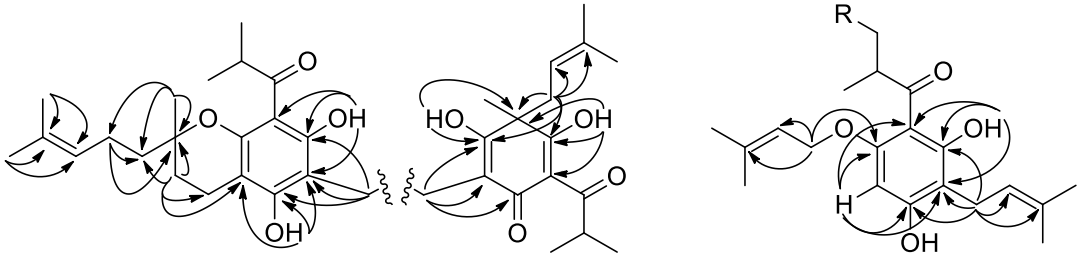

Fig. 3 Key HMBC correlations of hyperlaricifolin A (**2**) and laricifolin A/B (**3**/**4**)

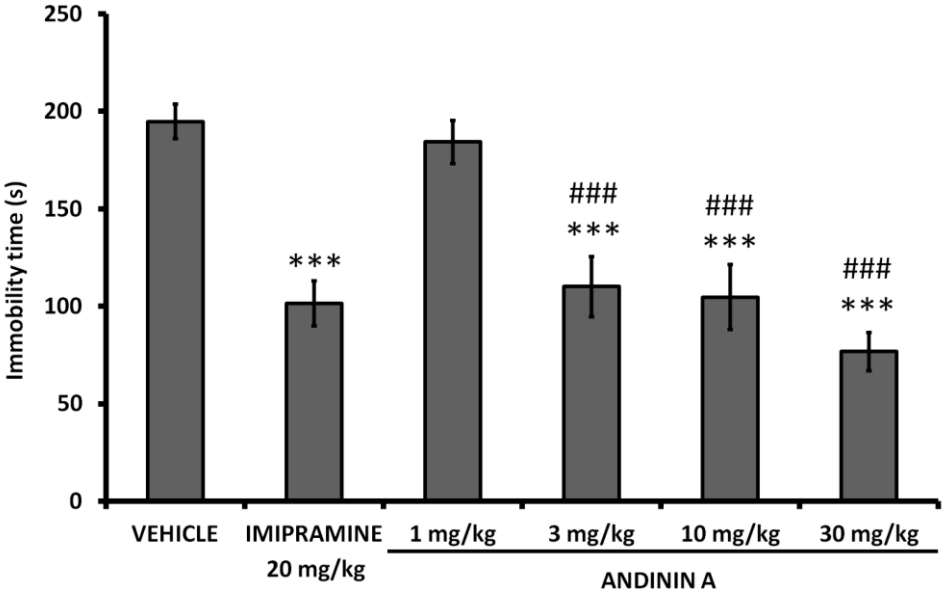

Fig. 4 Effect of different doses of andinin A (**1**), imipramine (20 mg/kg po), or vehicle (1 mL/100 g, po) in the mouse forced swimming test. The results are presented as means ± SEM (n = 8−10 mice/group). Significantly different values were detected by one-way ANOVA followed by a Student−Newman−Keuls test. ***$p < 0.001$ when compared to the vehicle group. $^{###}p < 0.001$ when compared to andinin A 1 mg/kg group.

Acylphloroglucinol derivatives from *Hypericum andinum* and *H. laricifolium*: antidepressant-like activity of andinin A

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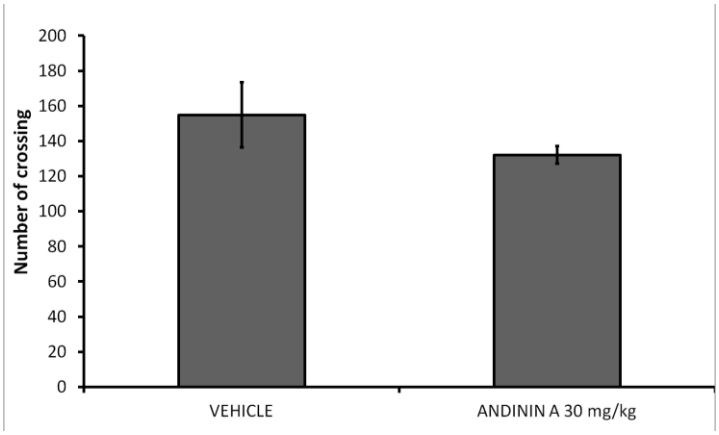
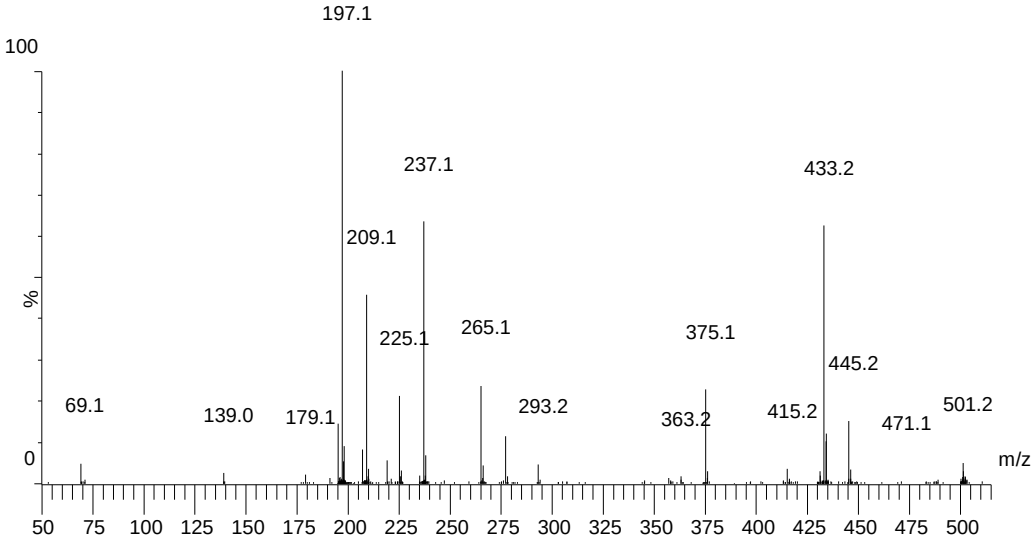
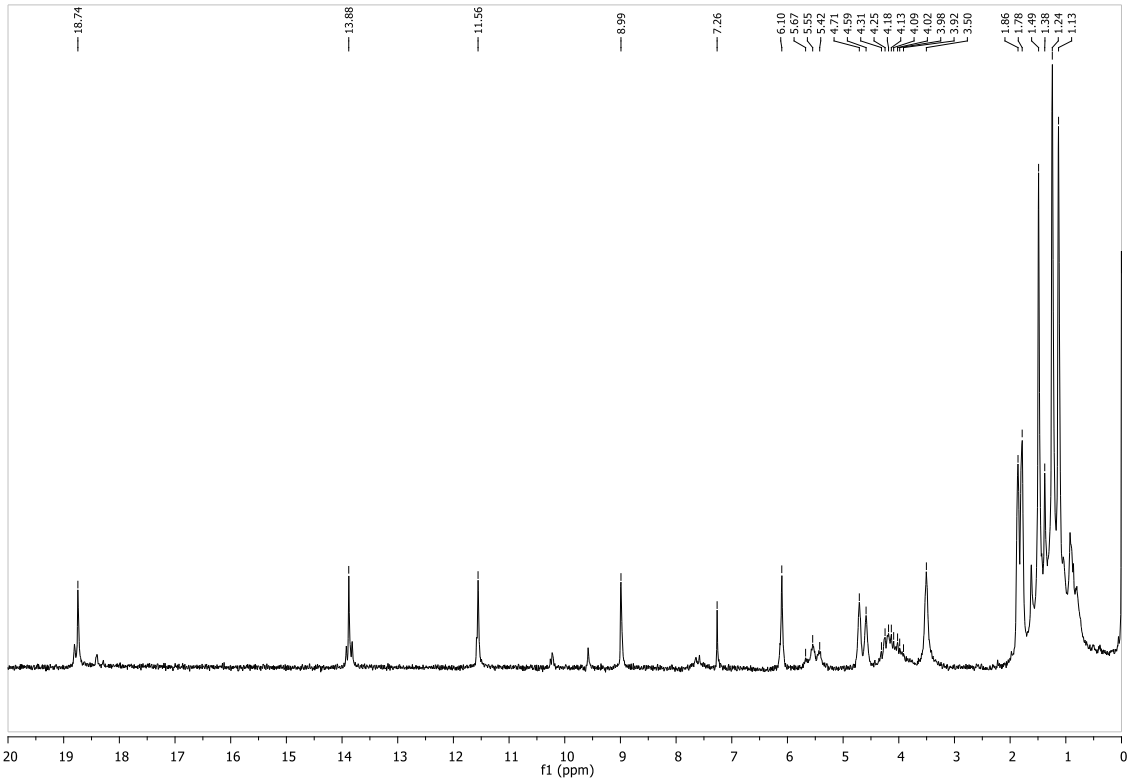
Supplementary information



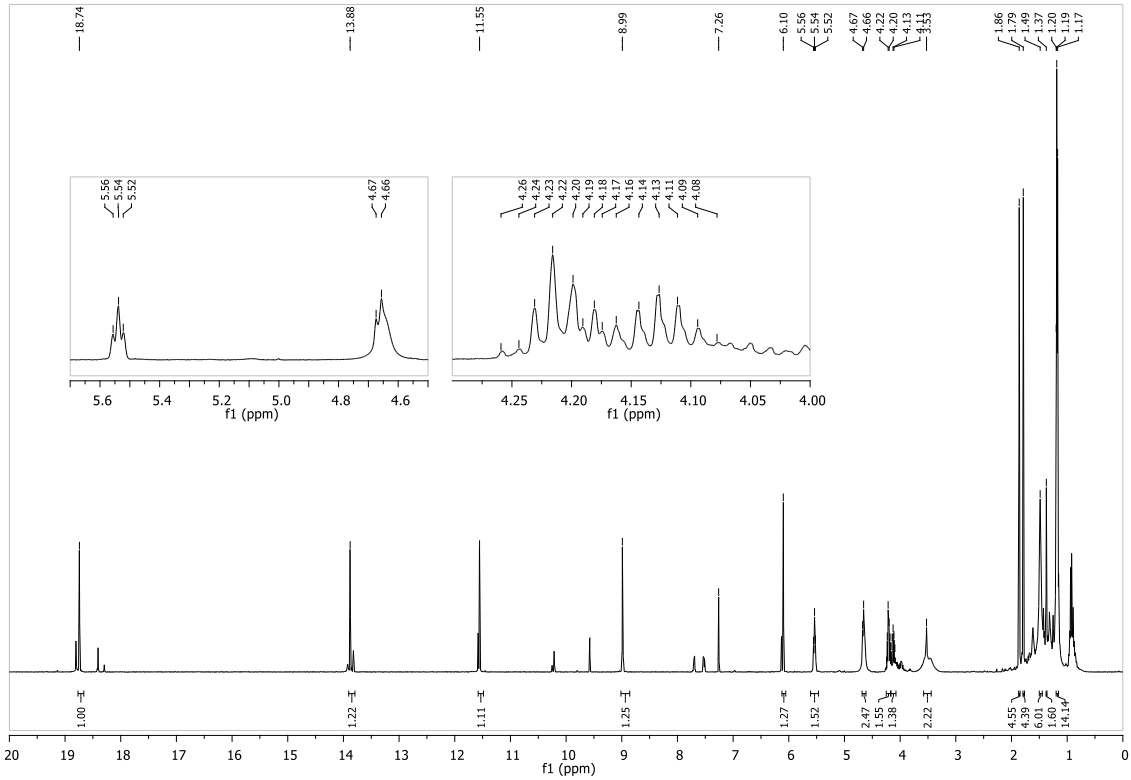
S1. Effect of acute treatment with andinin A (30 mg/kg, p.o.) or vehicle (p.o.) on the number of crossing in the mouse open field test. The results are presented as means ± SEM (n = 10 mice/group) and were compared by *t*-Student test.



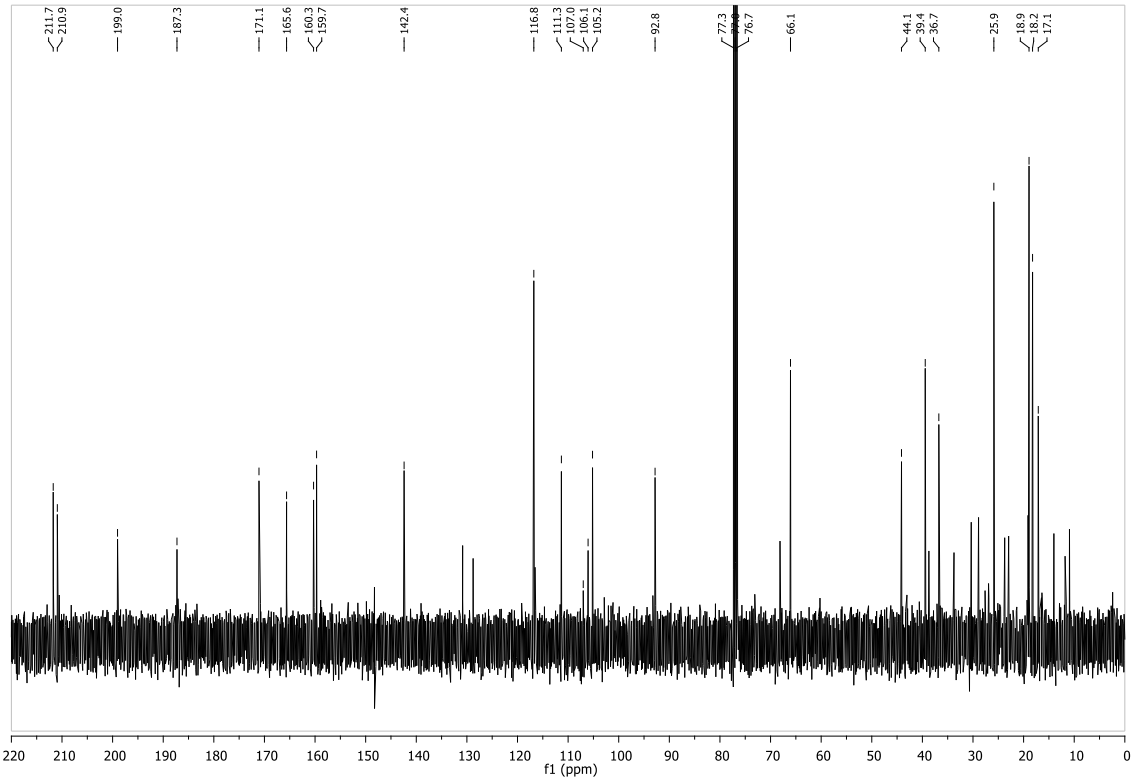
S2. ESIMS spectrum of andinin A (**1**)



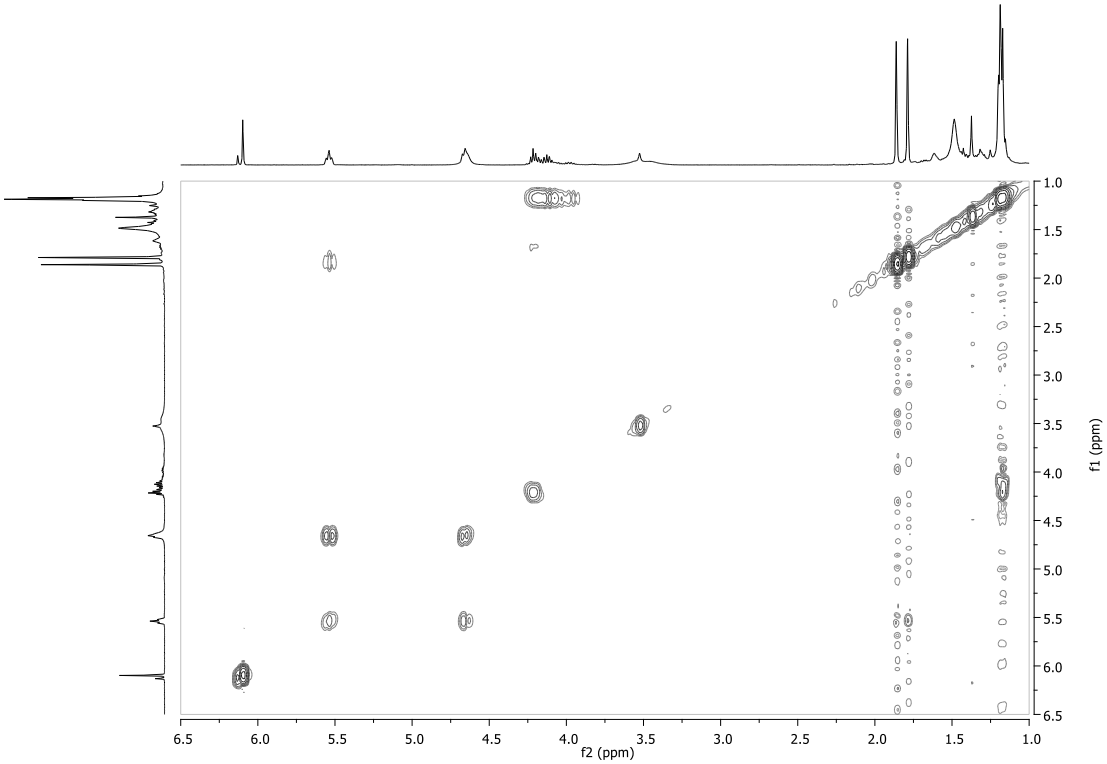
S3. ^{1}H NMR spectrum of andinin (**1**) ($CDCl_3$, 60 MHz)



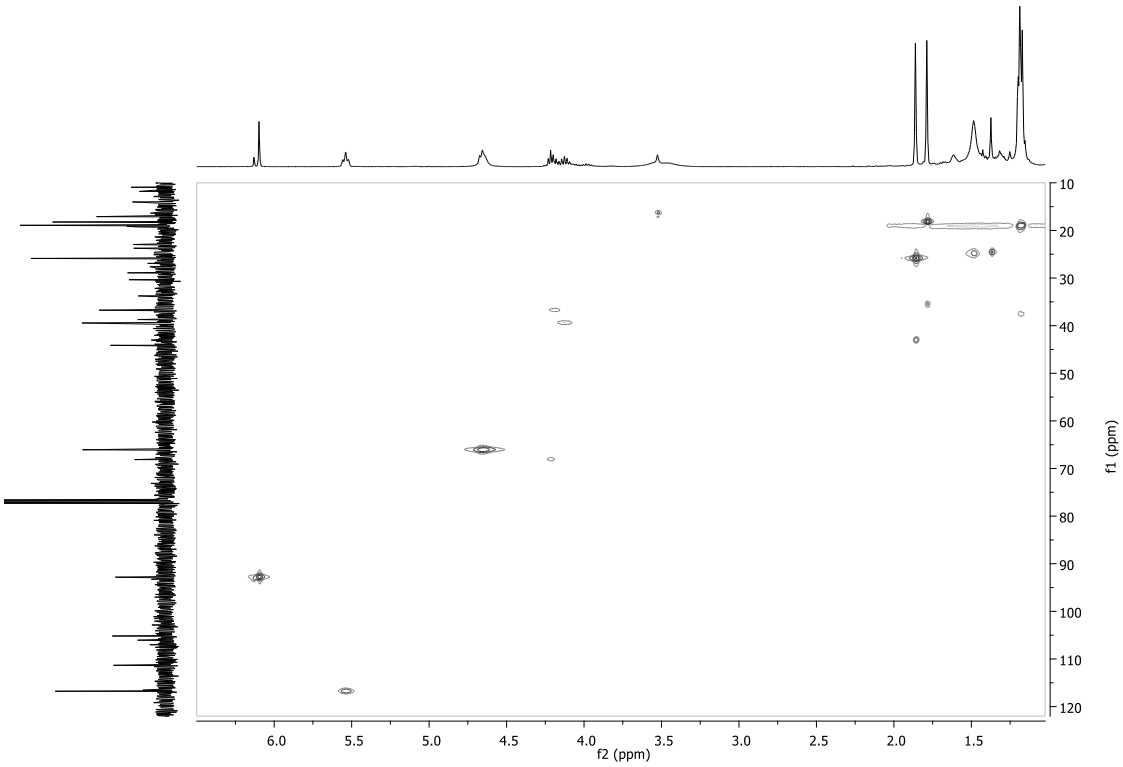
S4. ^{1}H NMR spectrum of andinin (**1**) ($CDCl_3$, 400 MHz)



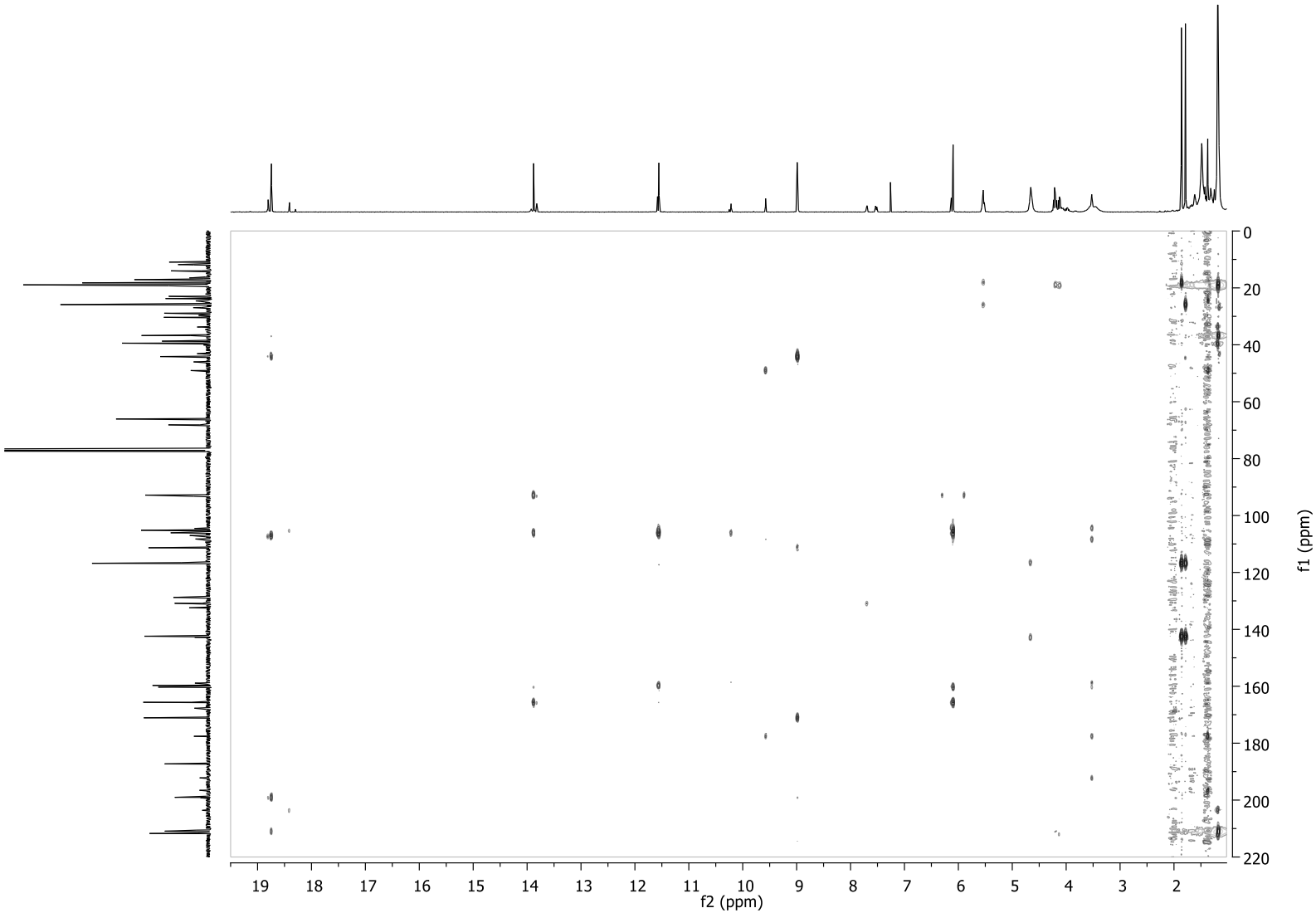
S5. ^{13}C NMR spectrum of andinin (**1**) ($CDCl_3$, 100 MHz)



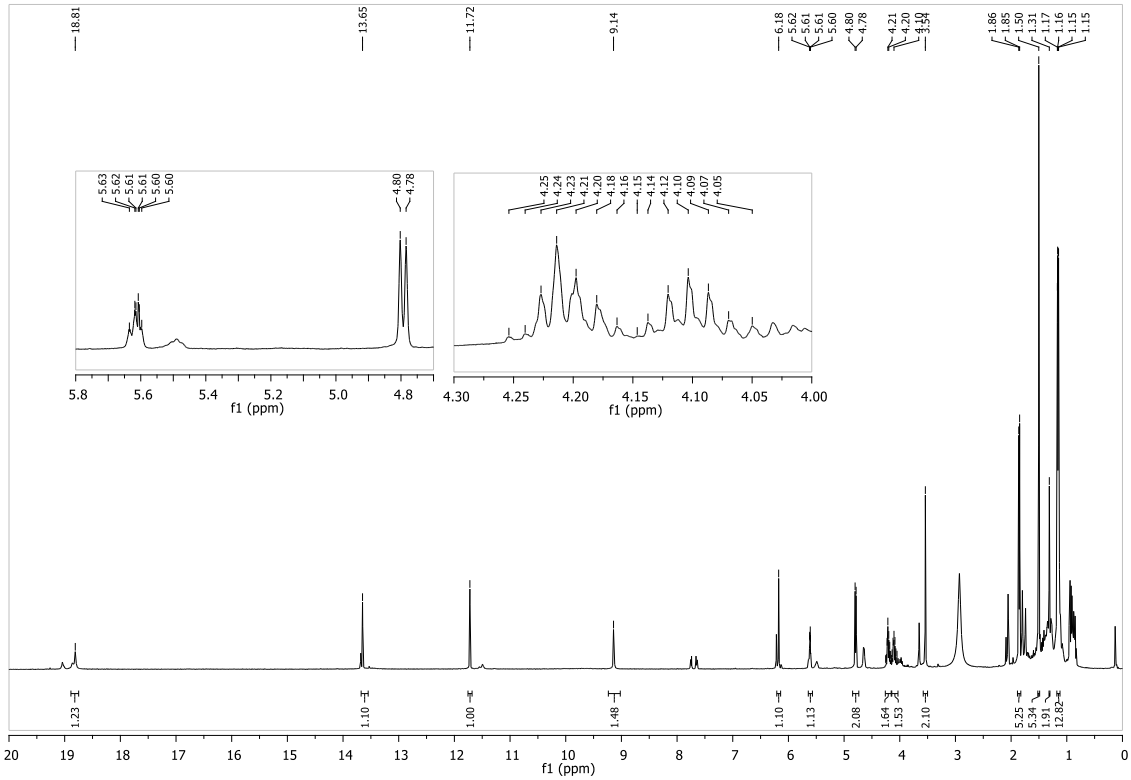
S6. ^{1}H-^{1}H COSY spectrum of andinin A (**1**) in $CDCl_3$



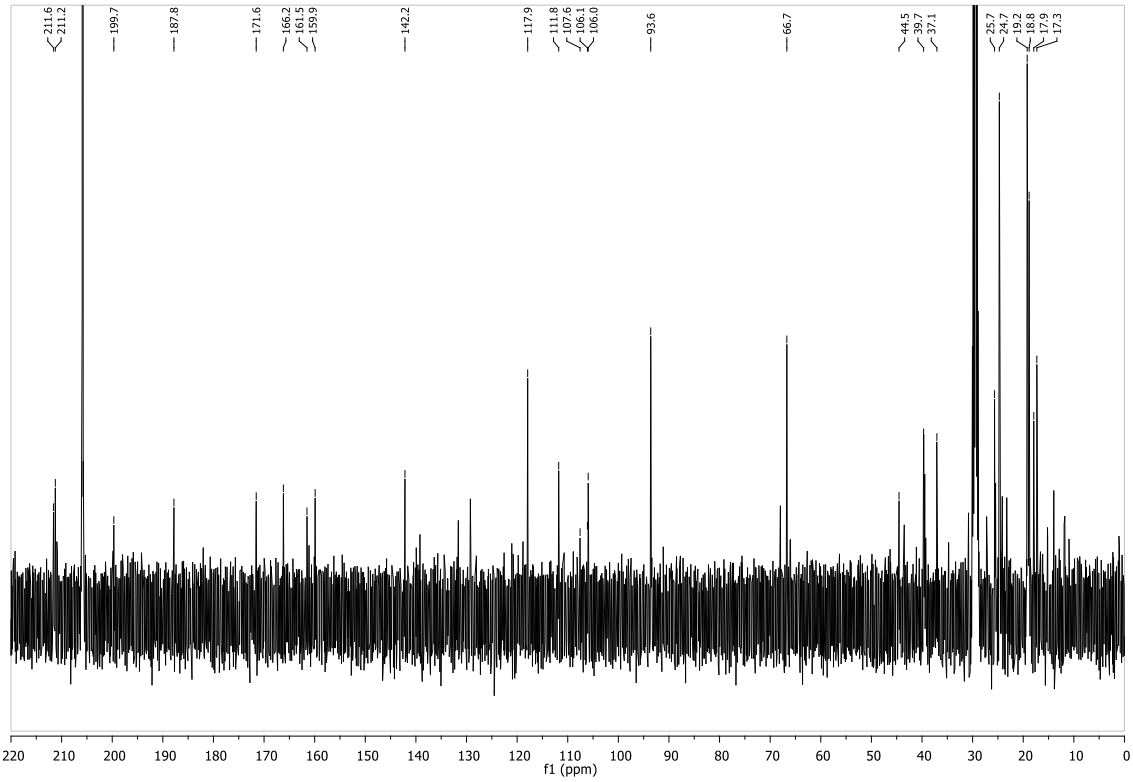
S7. HMBC spectrum of andinin A (**1**) in $CDCl_3$



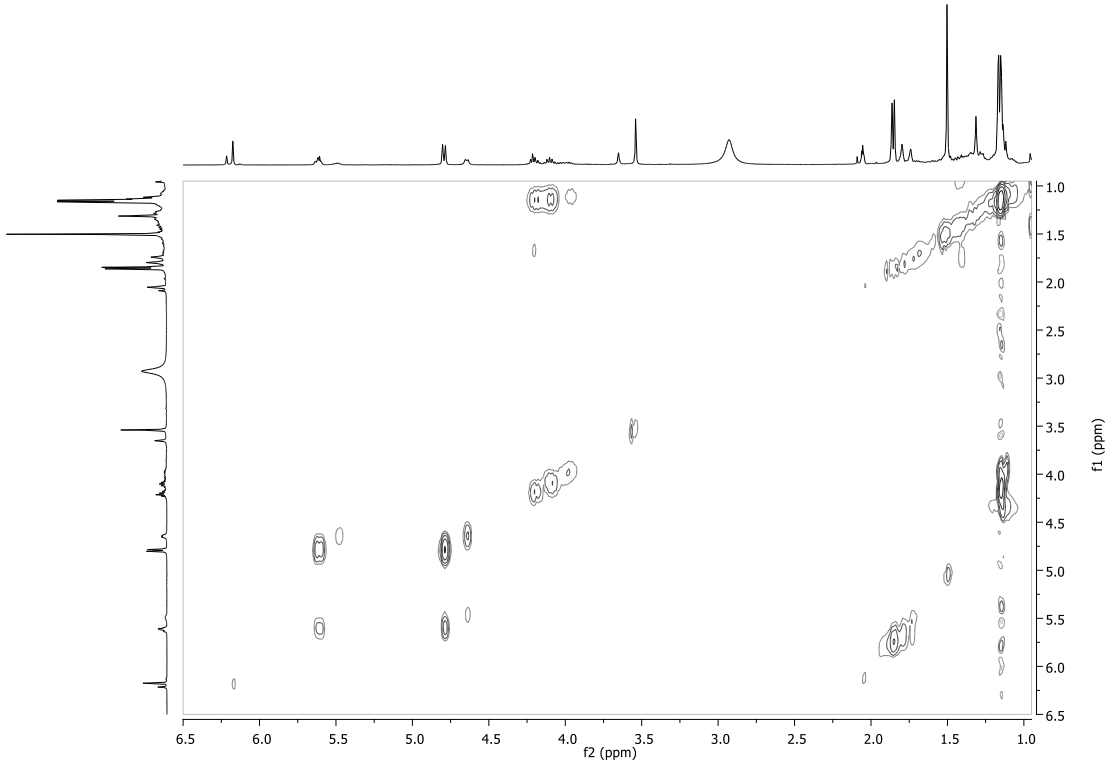
S8. HMBC spectrum of andinin A (**1**) in $CDCl_3$



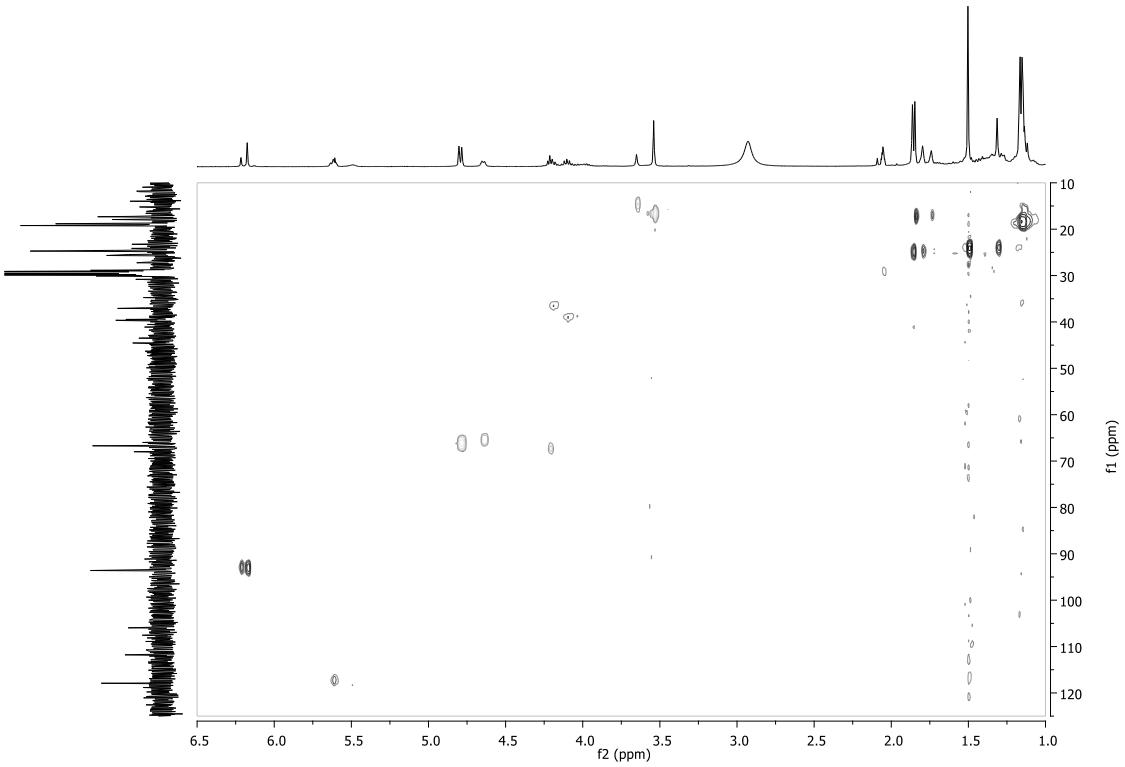
S9. ^{1}H NMR spectrum of andinin A (**1**) (acetone-d_6, 400 MHz)



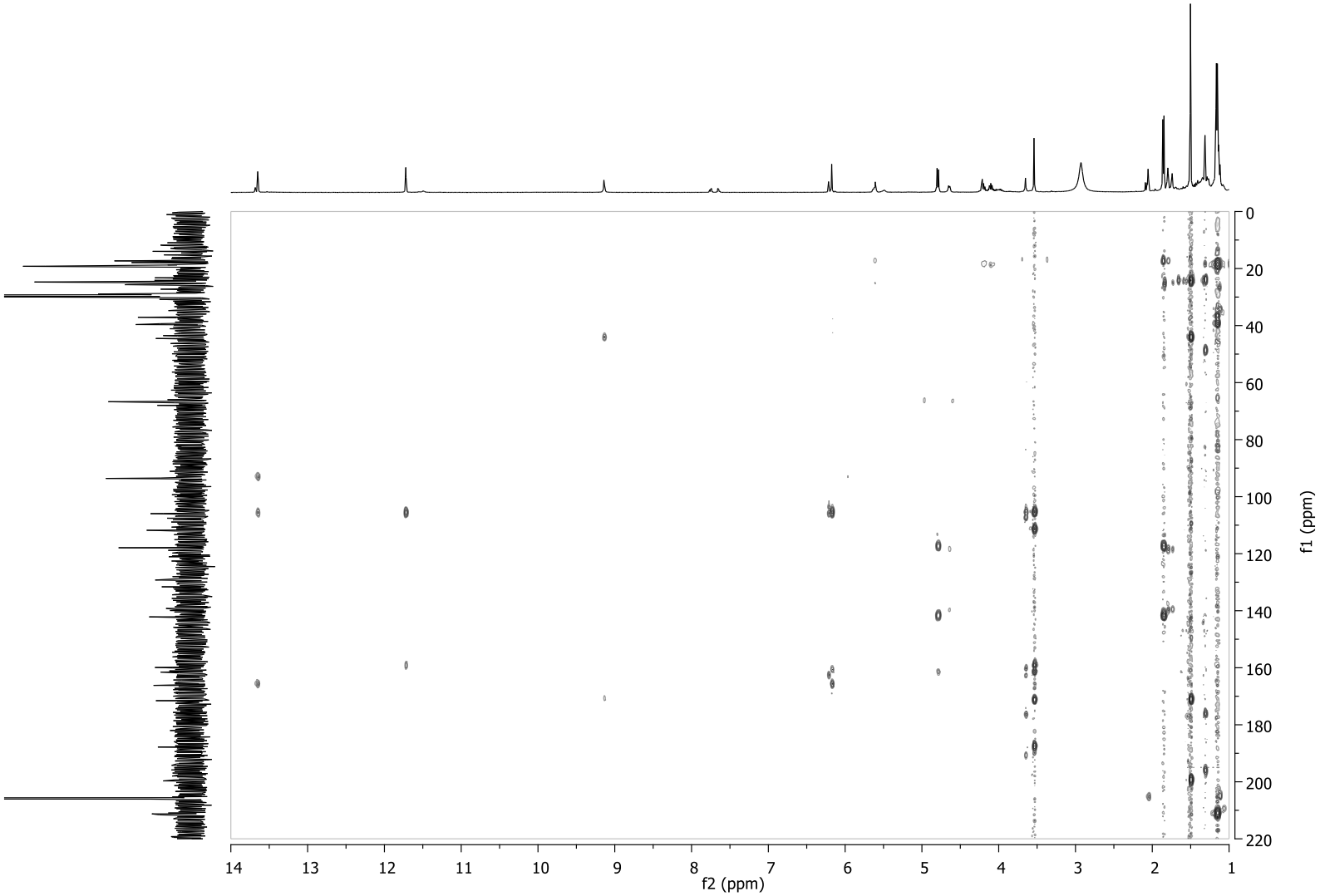
S10. ^{13}C NMR spectrum of andinin A (**1**) (acetone-d_6, 100 MHz)



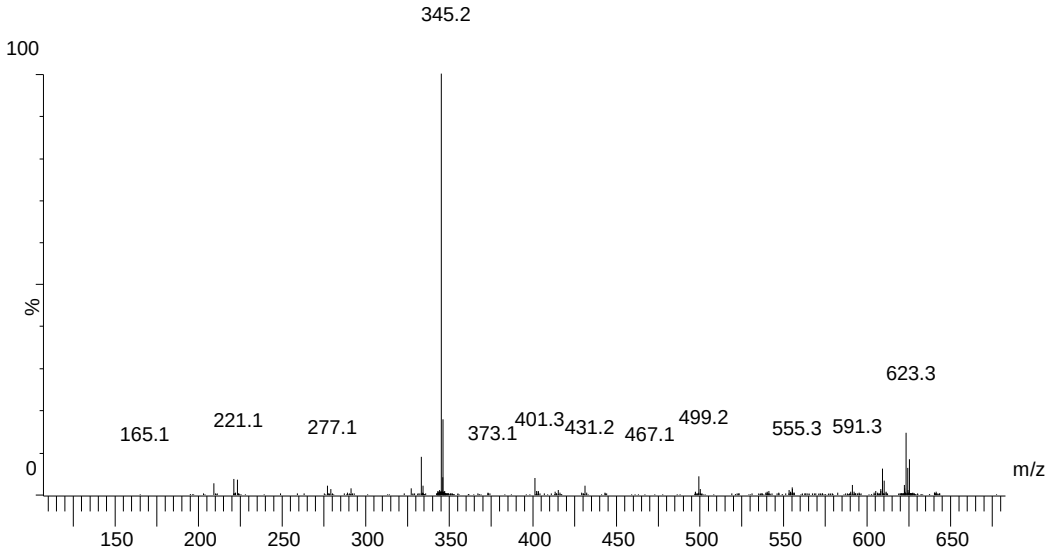
S11. ^{1}H-^{1}H COSY spectrum of andinin A (**1**) in acetone-d_6



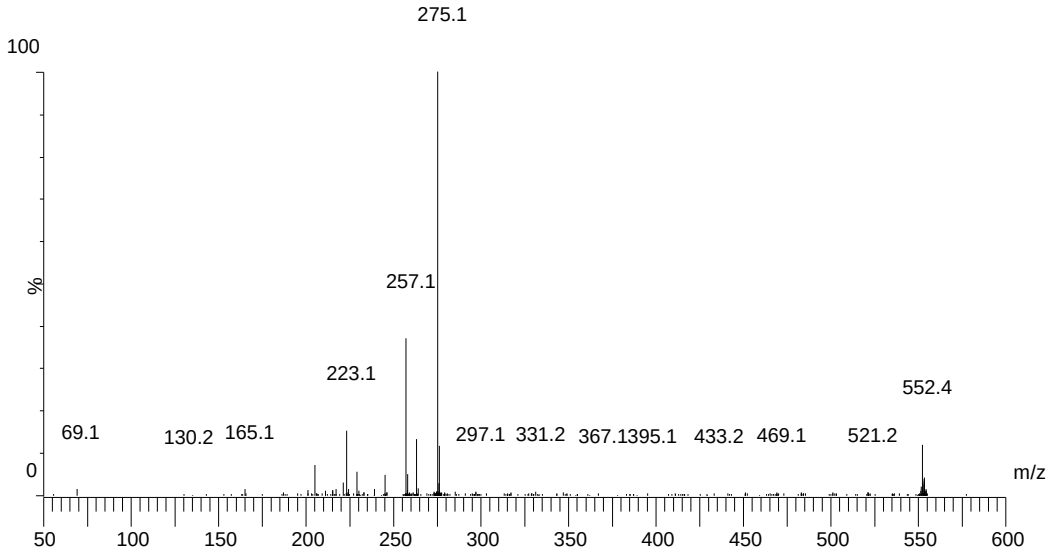
S12. HSQC spectrum of andinin A (**1**) in acetone-d_6



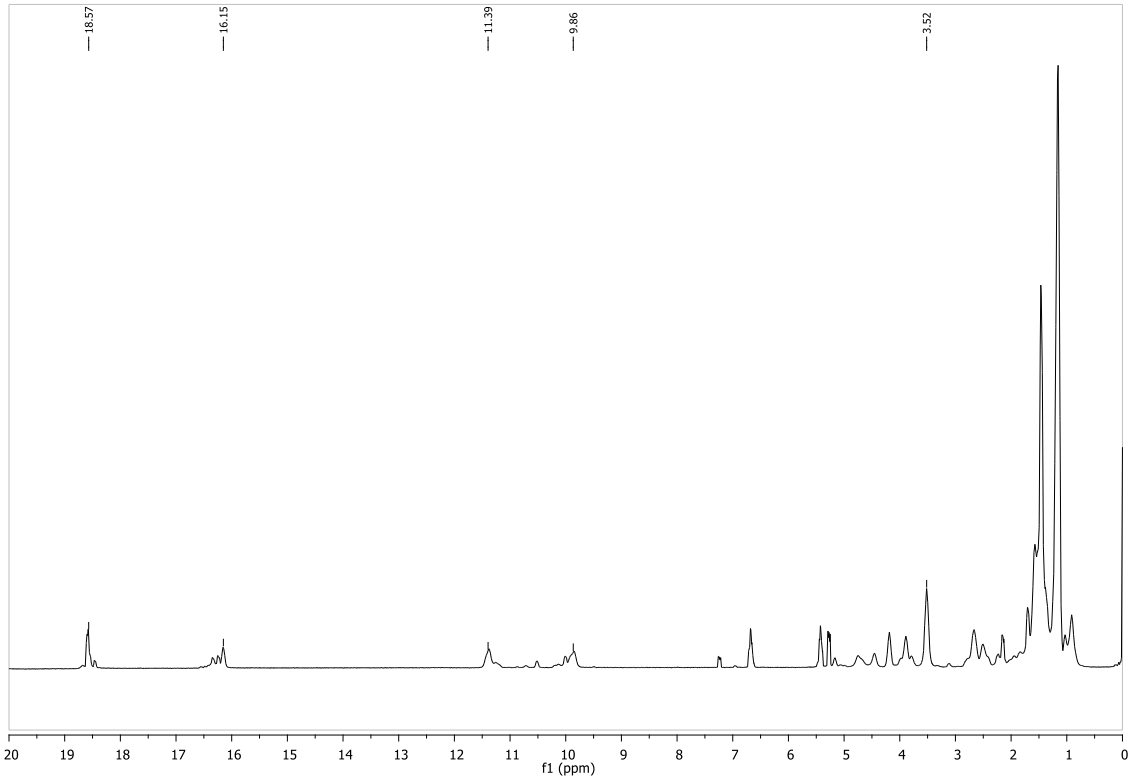
S13. HMBC spectrum of andinin A (**1**) in acetone-d_6



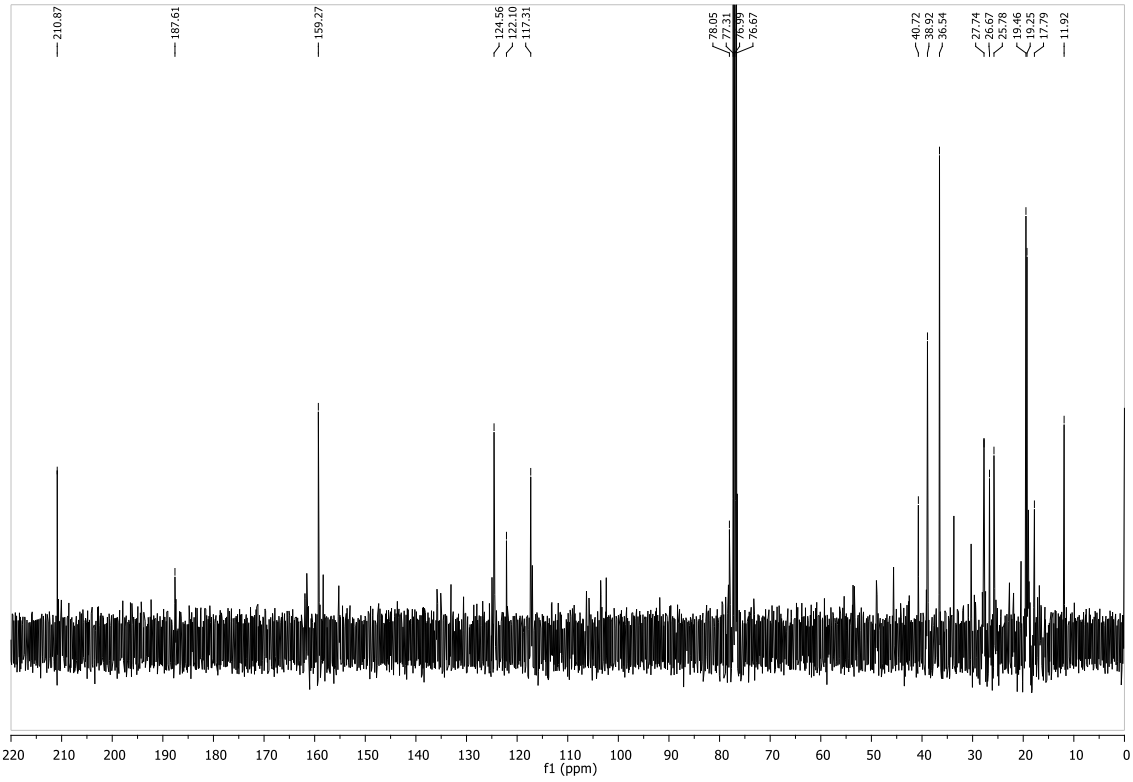
S14. ESIMS spectrum of hyperlaricifolin A (**2**)



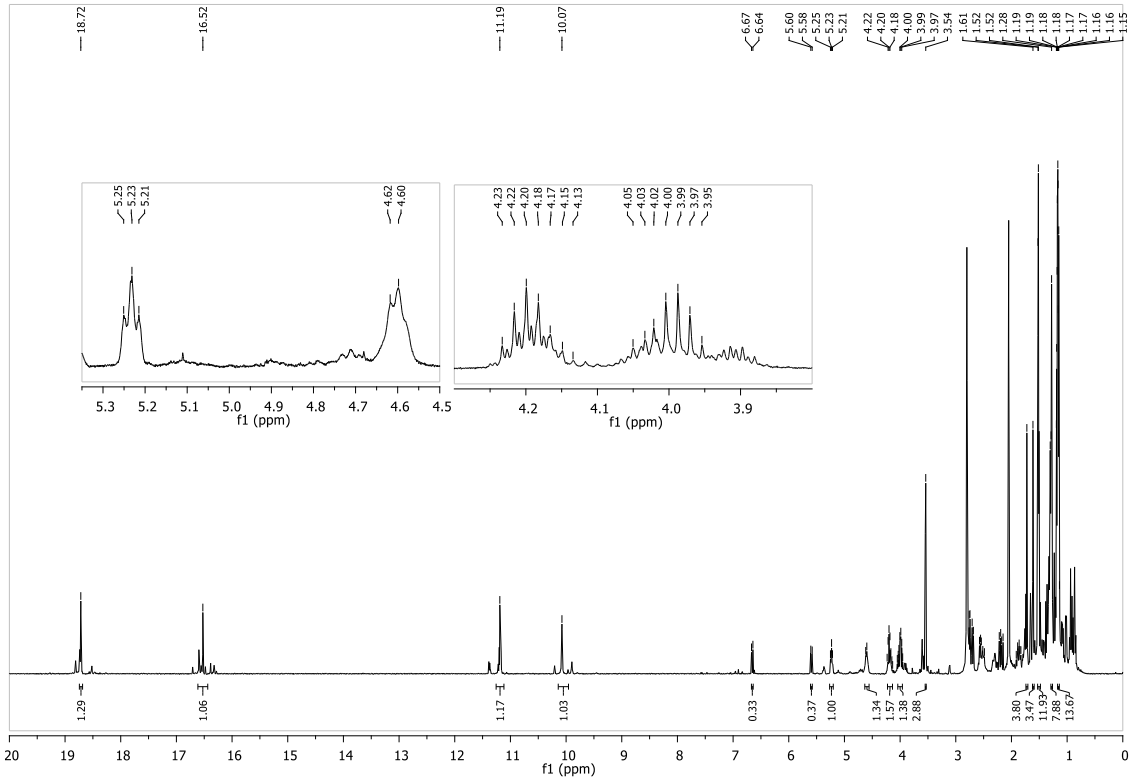
S15. ESIMS spectrum of hyperbrasilol B



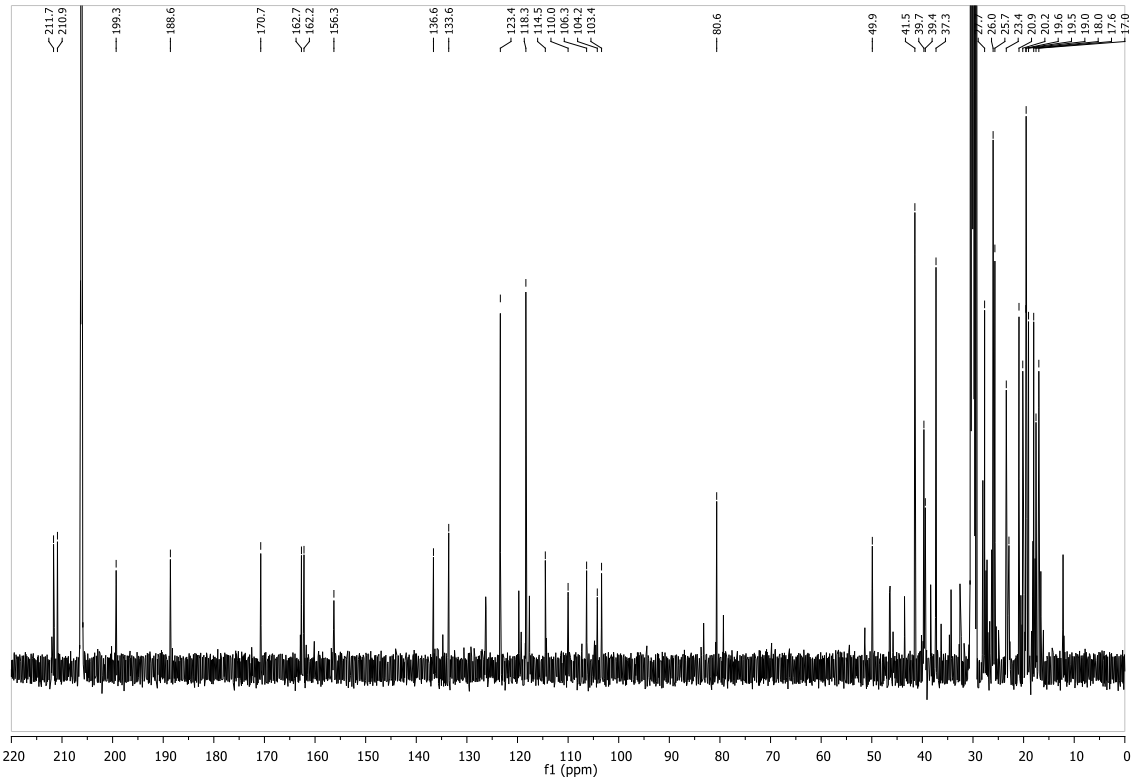
S16. ^{1}H NMR spectrum of hyperlaricifolin A (**2**) ($CDCl_3$, 400 MHz)



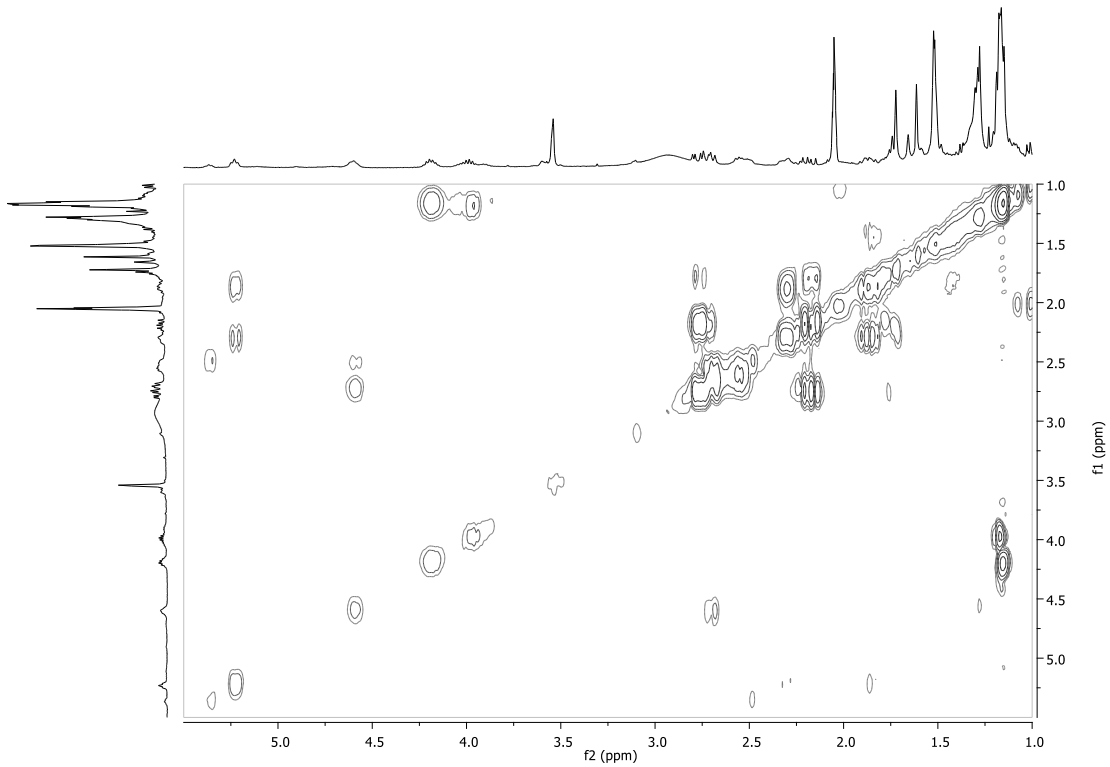
S17. ^{13}C NMR spectrum of hyperlaricifolin A (**2**) ($CDCl_3$, 100 MHz)



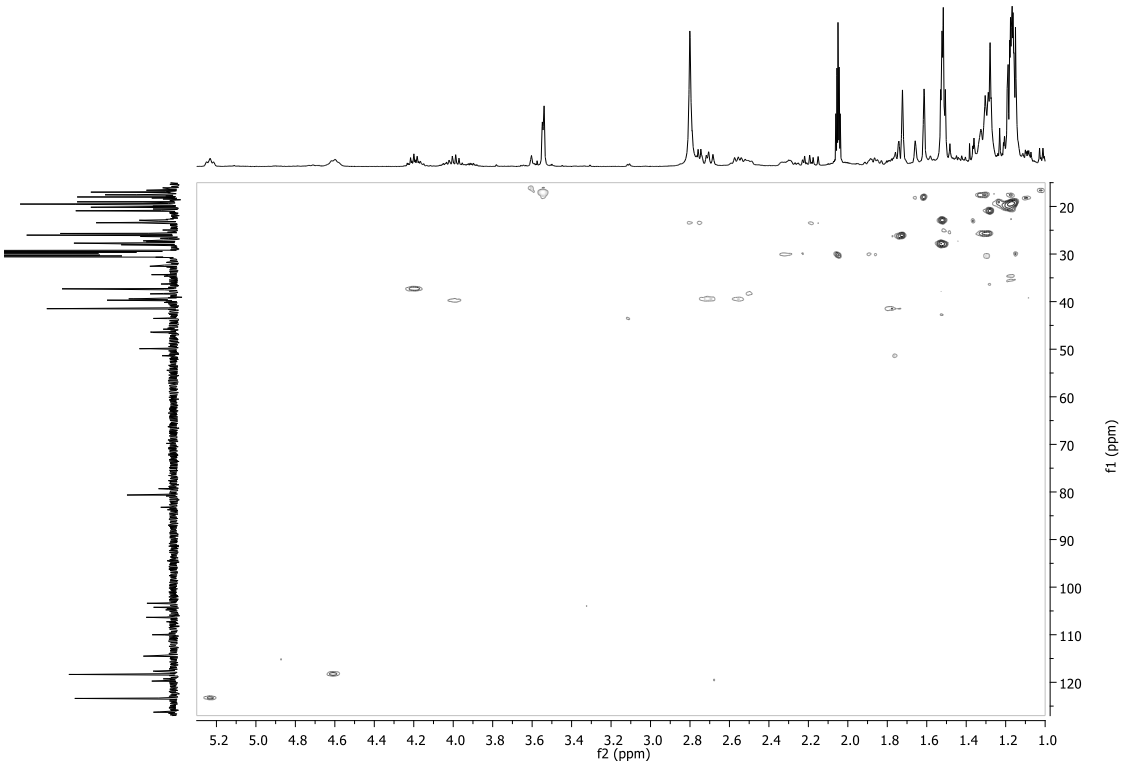
S18. ^{1}H NMR spectrum of hyperlaricifolin A (**2**) (acetone-d_6, 400 MHz)



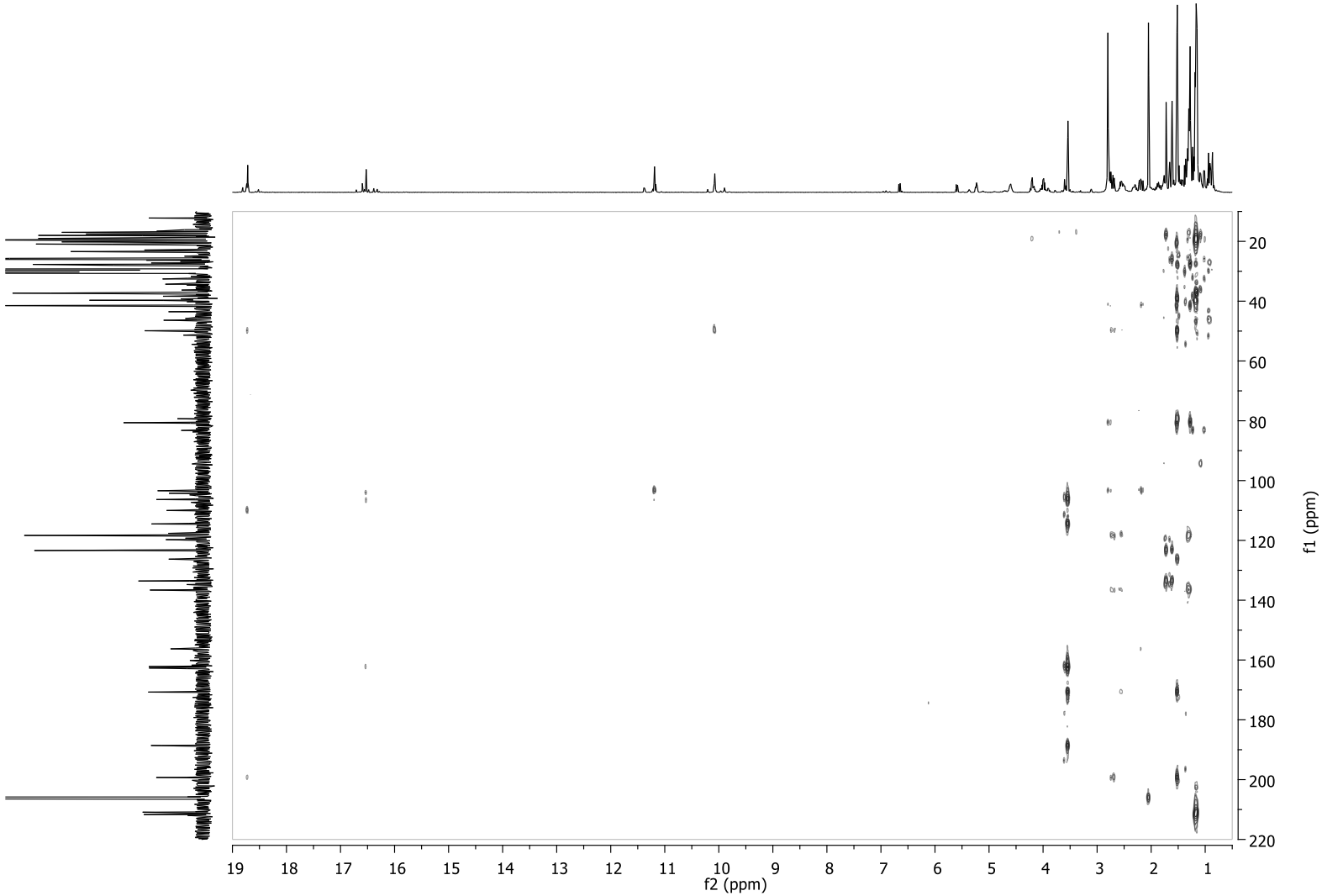
S19. ^{13}C NMR spectrum of hyperlaricifolin A (**2**) (acetone-d_6, 100 MHz)



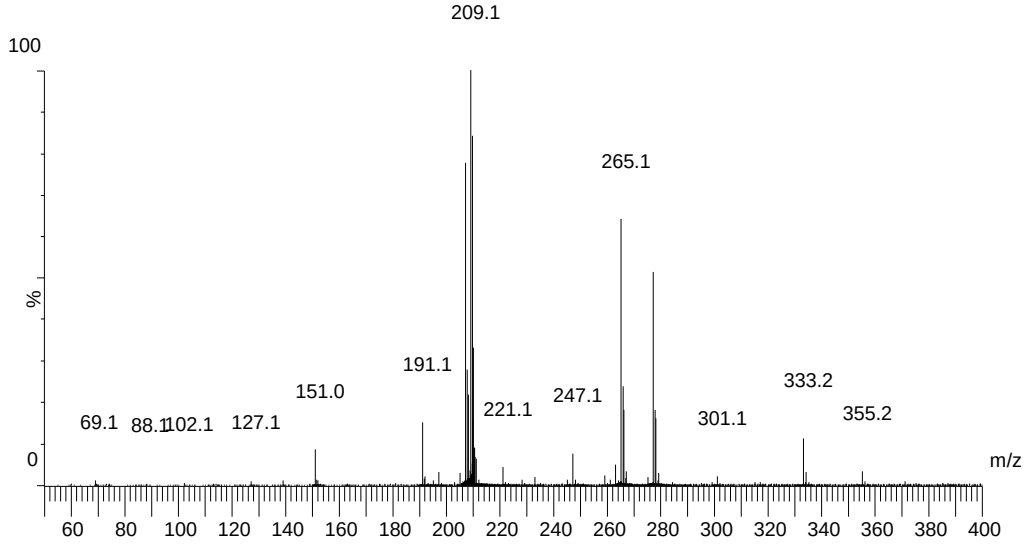
S20. ^{1}H-^{1}H COSY hyperlaricifolin A (**2**) in acetone-d_6



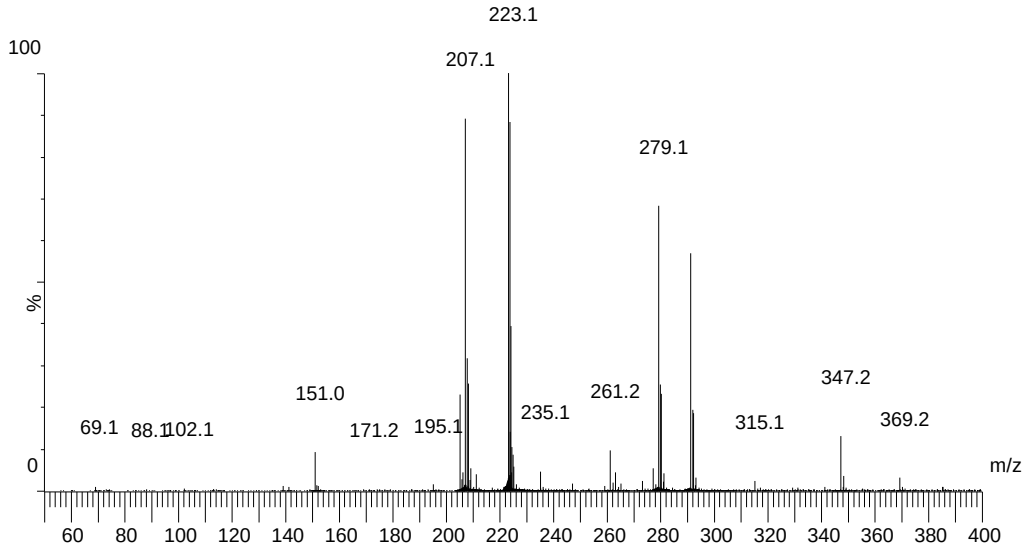
S21. HSQC spectrum of hyperlaricifolin A (**3**) in acetone-d_6



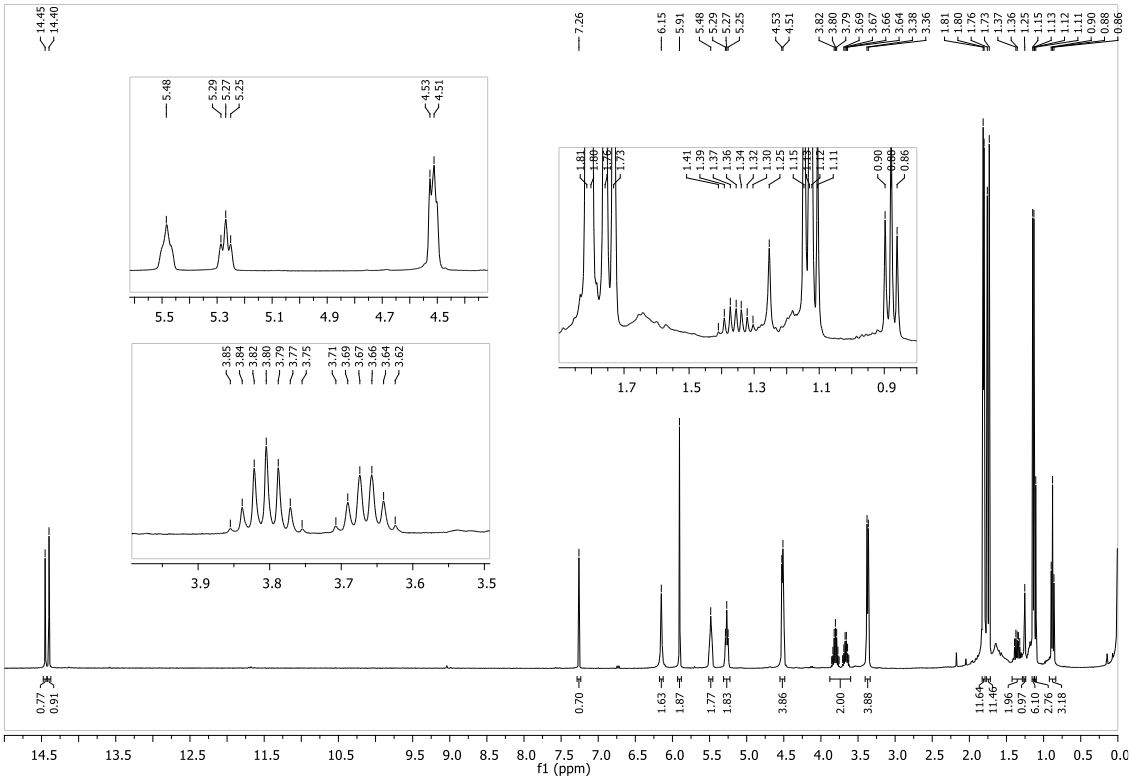
S22. HMBC spectrum of hyperlaricifolin A (**2**) in acetone-d_6



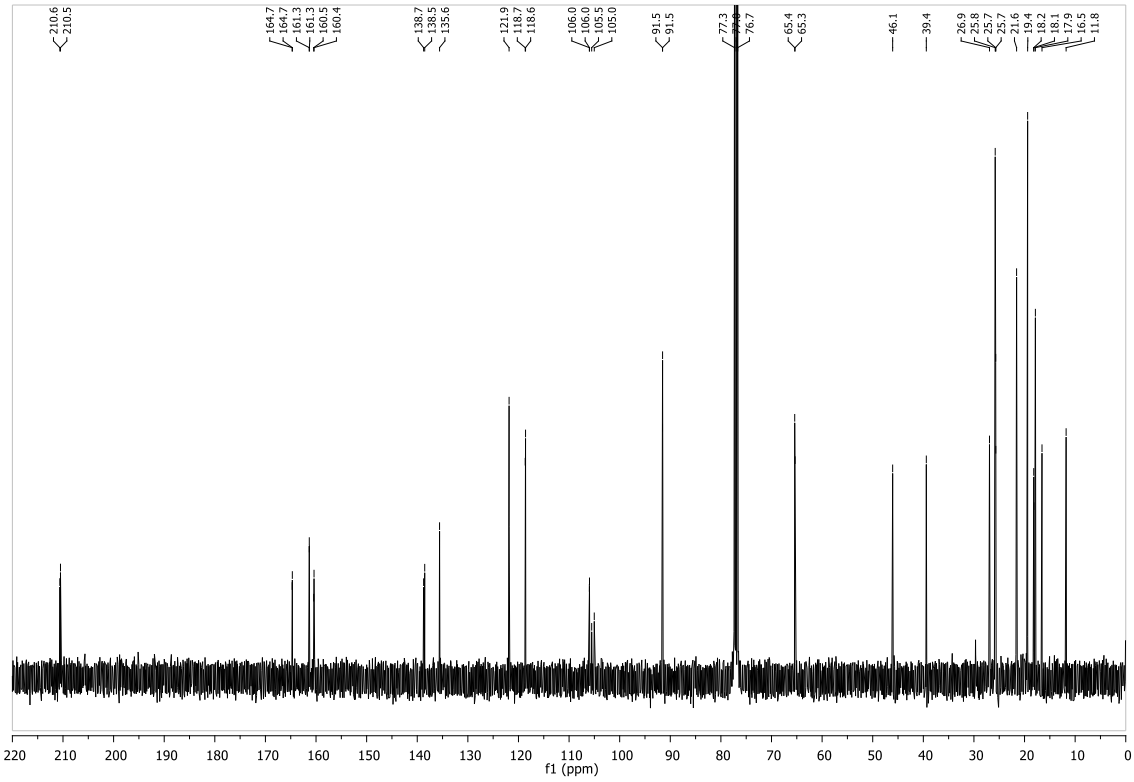
S23. ESIMS spectrum of laricifolin A (**3**)



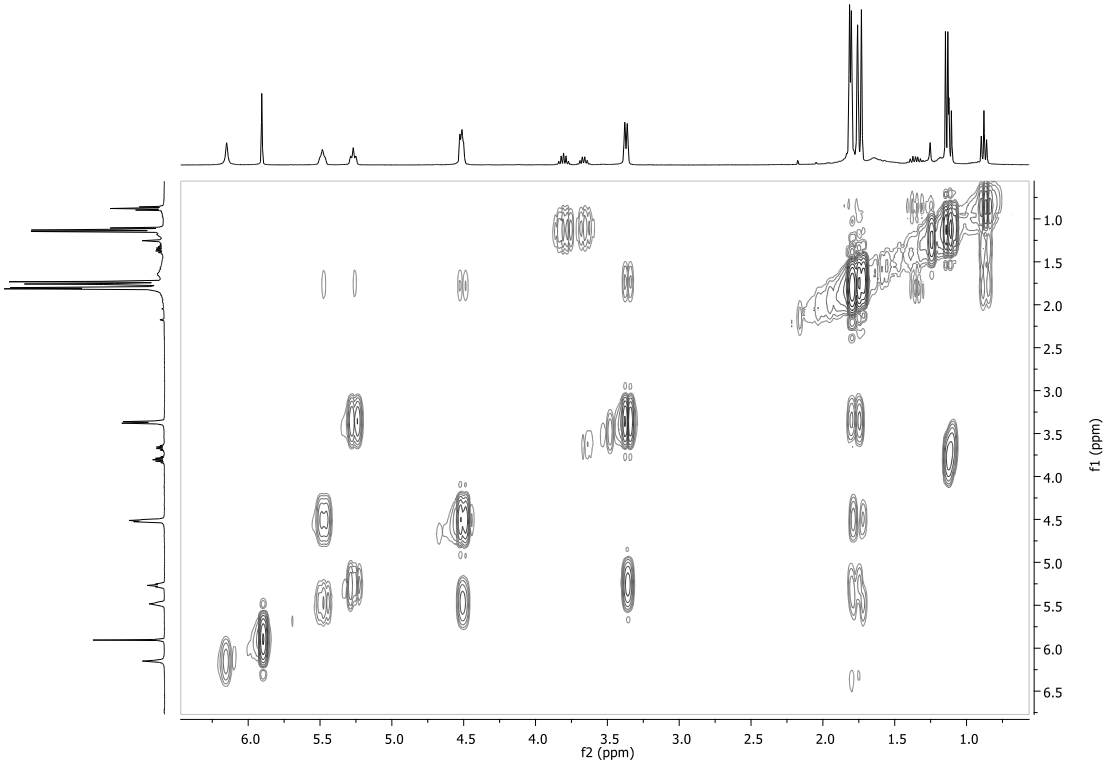
S24. ESIMS spectrum of laricifolin B (**4**)



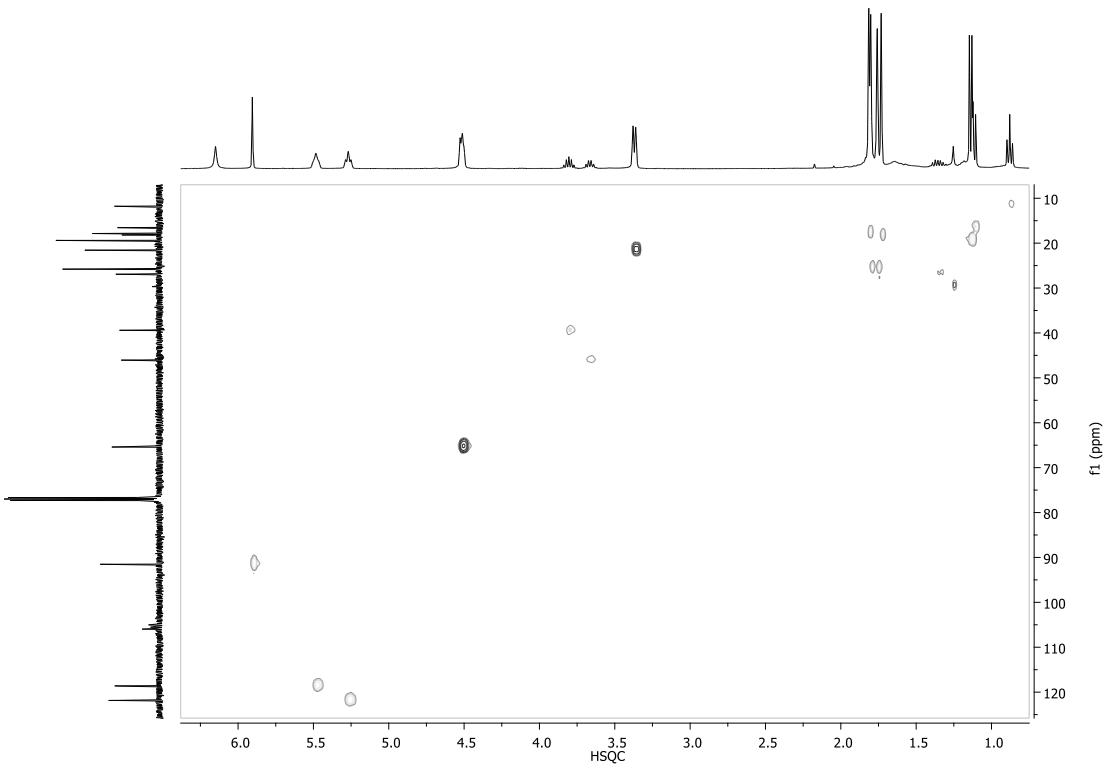
S25. ^{1}H NMR spectrum of laricifolin A (**3**) and laricifolin B (**4**) ($CDCl_3$, 400 MHz)



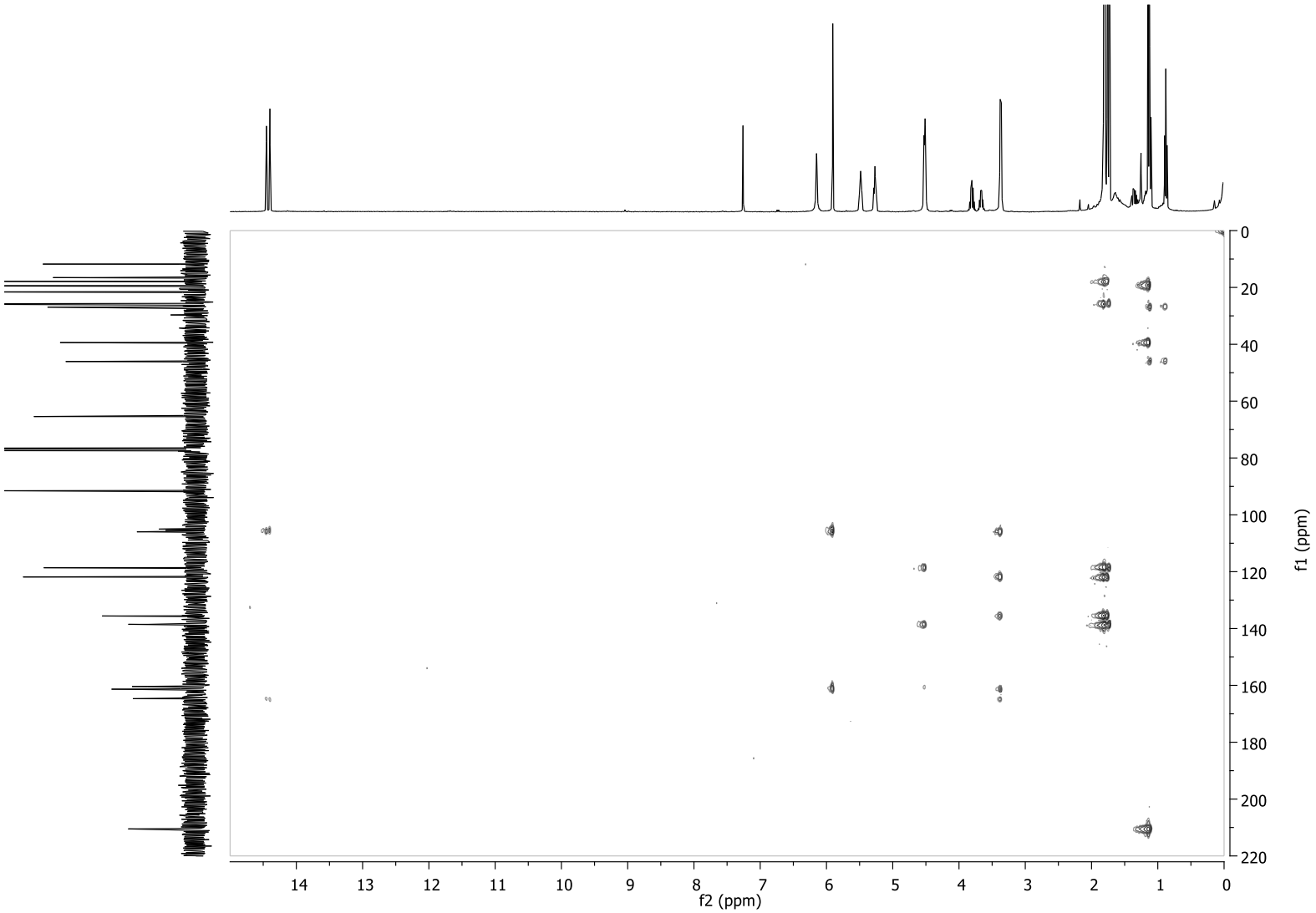
S26. ^{12}C NMR spectrum of laricifolin A (**3**) and laricifolin B (**4**) ($CDCl_3$, 100 MHz)



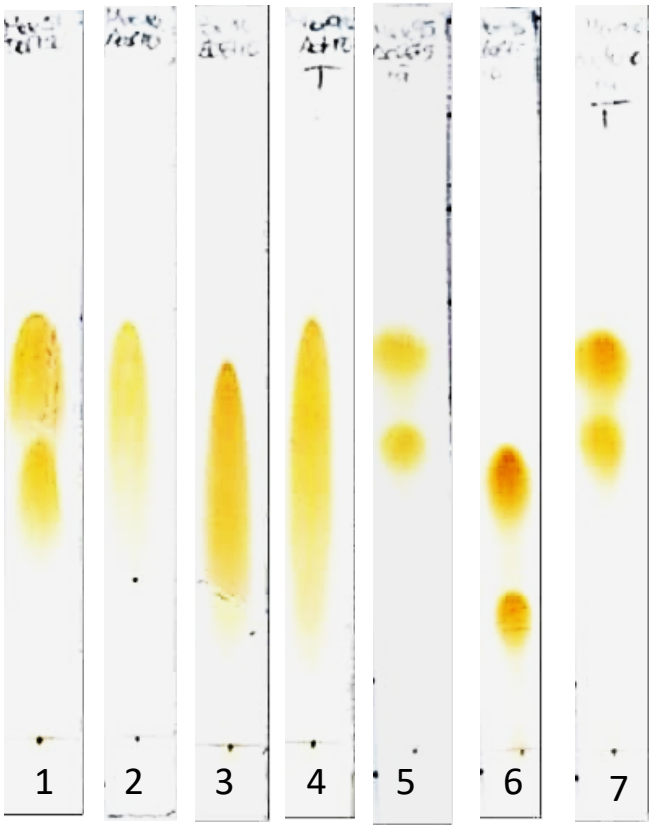
S27. ^{1}H-^{1}H COSY spectrum of laricifolin A (**3**) and laricifolin B (**4**) in $CDCl_3$



S28. HSQC spectrum of laricifolin A (**3**) and laricifolin B (**4**) in $CDCl_3$



S29. HMBC spectrum of laricifolin A (**3**) and laricifolin B (**4**) in $CDCl_3$



S30. Exemplification of mobile phase testing on a fraction of *Hypericum andinum* constituted by a mixture of isouliginosin B and andinin A previous to their final purification by Centrifugal planar chromatography (CPC) on a Chromatotron™ instrument. Mobile phase composition (**1**) Hex/DCM (50:50 v/v), (**2**) Hex/EtOAc (90:10 v/v), (**3**) Hex/EtOEt (95:5 v/v), (**4**) Hex/Acet (95:5 v/v), (**5**) Hex/EtOAc (95:5 v/v with 0.25 % formic acid), (**6**) Hex/EtOEt (97.5:2.5 v/v with 0.25 % formic acid) and (**7**) Hex/Acet (97.5:2.5 v/v with 0.25 % formic acid).

CAPÍTULO III

"Assessing the phytochemical profiles and antidepressant-like activity of four Peruvian *Hypericum* species using the murine forced swimming test"

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Assessing the phytochemical profiles and antidepressant-like activity of four Peruvian *Hypericum* species using the murine forced swimming test

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Summary

The antidepressant-like activity of crude ethanolic extracts from Peruvian *Hypericum* species (*H. andinum*, *H. brevistylum*, *H. silenoides* and *H. laricifolium*) was assessed using the forced swimming test (FST) in mice. Different doses of all extracts shortened the immobility time of mice in experimental groups, indicating that these extracts possessed a potential antidepressant-like activity. Phytochemical analyses revealed that the extracts were rich in flavonoids, principally hyperoside, and to a lesser extent, dimeric acylphloroglucinol derivatives. The phytochemical composition of these *Hypericum* species with reported antidepressant-like activity in the FST is briefly reviewed and the importance of flavonoids with reference to the activity highlighted.

Key words

Hypericum andinum, H. brevistylum, H. silenoides, H. laricifolium, hyperoside, Lorke acute toxicity test

Introduction

Extracts of *Hypericum perforatum* (St. John's wort) are used worldwide to treat mild to moderate depression. The spectrum of side effects is lower compared to that of current pharmacological treatments (Kasper et al. 2010). The naphthodianthrone hypericin, the polyisoprenylated phloroglucinol derivative hyperforin and several flavonoids appear to contribute to the antidepressant activity (Nahrstedt and Butterweck 2010).

The genus *Hypericum* (Hypericaceae) encompasses nearly 500 species occurring worldwide, and is currently divided into 36 taxonomic sections (Robson 2012). The majority of *Hypericum* species native to South America belong to the taxonomic sections *Brathys* and *Trigynobrathys* (Crockett et al. 2010). From these two sections, extracts from *H. caprifoliatum* and *H. polyanthemum* and their fractions have been the subject of several pharmacological studies, and demonstrated antidepressant and analgesic activity (Viana et al. 2003; Viana et al. 2006). Flavonoids and dimeric acylphloroglucinol derivatives were identified as mayor bioactive constituents in these extracts (Haas et al. 2010; Stein et al. 2012; Stolz et al. 2012).

Examinations and comparisons of chemical constituents and pharmacological profiles of various *Hypericum* species can be helpful in the search for alternative sources of antidepressant compounds (Viana et al. 2006). The aim of this study was to assess the potential antidepressant activity of crude ethanolic extracts of *H. andinum* Gleason, *H. brevistylum* Choisy, *H. laricifolium* Juss and *H. silenoides* Juss, as well as to identify their mayor phytochemical components. The phytochemical composition of these *Hypericum* species with antidepressant-like activity in the FST is reviewed and the importance of flavonoids with reference to the activity highlighted.

Materials and Methods

Chemicals and plant materials

The reference substances chlorogenic acid (**1**), rutin (**2**), guaijaverin (**4**) and quercetin (**5**) were purchased from Sigma (USA, purity ≥ 95%). Hyperoside (**3**) was isolated by chromatographic methods (purity ≥ 94%) from aerial parts of *H. andinum* as previously described (Bernardi 2007). The dimeric acylphloroglucinols uliginosin A (**6**), isouliginosin B (**7**), isohyperbrasilol B (**8**), uliginosin B (**9**) and hyperbrasilol B (**10**) were isolated from *H. andinum* Gleason, *H. brevistylum* Choisy, *H. laricifolium* Juss and *H. silenoides* Juss as described elsewhere (Ccana-Ccapatinta et al. 2013), with purities of 75, 70, 91, 74 and 90%, respectively. Fluoxetine-HCl was obtained from CFR Pharmaceuticals (Peru, Purity ≥ 99%). The identity and purity of the isolated compounds were confirmed by HPLC analysis and, ^{1}H and ^{13}C NMR spectroscopy.

Aerial parts in blossom of the *Hypericum* spp. listed in **Table 1** were collected from several Peruvian Páramo habitats characterized by their high altitude. A collection permit (0147-2010-AG-DGFFS-DGEFFS) was issued by the Dirección de Gestión Forestal y de Fauna Silvestre and Dirección General Forestal y de Fauna Silvestre of the Republic of Peru. Voucher specimens have been deposited in the Vargas CUZ herbarium at the Universidad Nacional de San Antonio Abad del Cusco (UNSAAC) and were identified by the botanist Washington H. Galiano Sánchez (Academic Department of Biology, UNSAAC). The plant material was dried at room temperature and ground to powder prior to extraction.

Preparation of extracts

Extracts were obtained by maceration of the plant material (plant/solvent ratio = 1/10) with 96% ethanol for 24 hrs. The extracts were filtered and concentrated *in vacuo* at 45°C, then stored at 4 ºC until test and analyses were performed. This extraction process was repeated three times, and the concentrated extracts of each species combined. The yields (w/w) obtained were 35.6% for *H. andinum*, 28.3% for *H. brevistylum*, 24.6% for *H. laricifolium* and 23.1% for *H. silenoides*. A portion of each extract was suspended in methanol at a concentration of 2.5 mg/mL and filtered (0.22 µm pore size, Sartorius) prior to HPLC analysis.

Phytochemical characterization of extracts

Thin layer chromatography (TLC)

The extracts were screened for flavonoids and naphthodianthrones by TLC following Wagner and Bladt 1996, and Maleš et al. 2004. TLC was performed in triplicate on aluminum sheets precoated with silica gel 60 F_{254} (Merck, Darmstadt, Germany) using ethyl acetate/formic acid/glacial acetic acid/water - 100:11:11:26 v/v as the mobile phase. After development, the chromatograms were air-dried and subsequently sprayed with natural products reagent (1% of diphenylboryloxyethylamine in methanol, w/v), then visualized under UV light (365 nm).

High performance liquid chromatography (HPLC)

An initial HPLC-DAD (diode array detector) analysis of each extract was carried out following the method described in Ganzera et al. 2002. Briefly, separations were performed on a Synergi MAX-RP 80Å column (4 μm 2.1 × 150 mm) from Phenomenex (Torrance, CA, USA) using an Agilent 1100 Separation Module (Agilent Technologies, Palo Alto, CA, USA). The mobile phases consisted of a mixture of 10 mM ammonium acetate buffer adjusted to pH 5.0 with glacial acetic acid (A) and a 9:1 mixture of acetonitrile and methanol (B). Gradient elution was performed as follows: linear gradient from 13% B to 17% B over 10 min, then increasing to 100% B over 25 min, followed by re-equilibration for 7 min. All separations were performed at 40°C at a flow rate of 0.3 mL/min. An aliquot of 10 μL of extract per sample was injected.

Additional HPLC analyses were carried out following a method described in Tatsis et al. 2007 with some modification. Separations were performed on a Waters Nova-Pack C18 column (4 μm, 3.9 mm × 150 mm) adapted to a guard column Waters Nova-Pack C18 60Å (Waters, Milford, MA, USA) using a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan). The mobile phases consisted of a mixture of water (A) and a 8:2 mixture of acetonitrile and methanol (B), both acidified with 0.1% formic acid. Gradient elution was performed as follows: linear gradient from 10% B to 100% B over 30 min, held at 100% B for 20 min, followed by re-equilibration for 20 min. All separations were performed at 25°C at a flow rate

of 1.0 mL/min, with detection performed at 220 and 350 nm, in triplicate. An aliquot of 10 µL of extract per sample was injected. By comparing retention times (t_R) of standard peaks with sample peaks, the peak identity of flavonoids (t_R 7.51, 12.52, 12.77, 13.41, 16.59 min for **1**, **2**, **3**, **4** and **5** respectively) and dimeric acylphloroglucinols (t_R 29.05, 37.66, 39.09, 39.56 and 40.81 min for **6**, **7**, **8**, **9** and **10** respectively) were assigned. In cases of uncertainty, samples were additionally spiked with standard compounds to confirm assignments.

The relative concentrations of the flavonoids and dimeric acylphloroglucinols were quantified using calibration curves prepared with **3** and **10** dissolved in methanol with six data points covering the concentration range of 1–500 µg/mL. These solutions were injected in triplicate, on three different days. The calibration curves were obtained by plotting the peak area signals as a function of concentration. Linearity was evaluated by least-squares regression analysis (r^2 = 0.9997 for **3**; r^2 = 0.9996 for **10**).

Ultra performance liquid chromatography (UPLC)

The phytochemical profiles of the extracts were further characterized by using UPLC-DAD/Q-TOF-MS with a Waters Acquity UPLC system connected to a Waters Q-TOF Premier mass spectrometer (Waters, Milford, CA, USA). The ACQUITY UPLC® Columns calculator (Waters, Milford, CA, USA) was used to modify the adapted HPLC method of Tatsis et al. 2007, described above, into a UPLC method. Separations were performed on a Hypersil Gold C18 column (1.9 µm, 2.1 mm x 100 mm). The mobile phases consisted of water (A) and a 8:2 mixture of acetonitrile and methanol (B), both acidulated with 0.1% formic acid. Gradient elution was performed as follows: linear gradient from 0% B to 100% B for the first 10 min, held at 100% B over the next 2 min, then re-equilibrated for 3 min. All separations were performed at 45°C and column flow rate was 0.6 mL/min. An aliquot of 4 µL of extract (500 µg/ml) per sample was injected. Mass detection was carried out in positive mode from *m/z* = 50 to 1000 and data were processed using MassLynx V4.1 software. Nitrogen and argon were used as the nebulizer and collision gases, respectively. Other MS detection condition were as follows: ESI capillary voltage, +3.0kV; source and desolvation temperatures, 120 and 300°C, respectively; desolvation and cone gas flows, 380 and 50 L/h, respectively; sample cone voltage and collision energy, 30V and 4 eV, respectively.

Animals

Male albino Balb/c/CNPB mice (20-24 g) were purchased from the Instituto Nacional de Salud (INS, Lima, Peru) and were housed in groups of 20 animals per cage (30 x 25 x 15 cm) in the Centro de Estudios de Plantas Alimenticias y Medicinales laboratory (CEPLAM, Cusco, Peru) two weeks prior to pharmacological studies. Animals were maintained in a temperature-controlled environment (20 ± 2°C). INS bioterium food and water were freely available. Subjects were experimentally naïve and used only once. All animals were subjected to fasting overnight before dosing. Plant extracts and the standard drug were suspended in a 2% aqueous solution of polysorbate 80, when were given orally 1 h before the experiments in a dose volume of 0.5 ml/20 g mice body weight. Control animals received 2% polysorbate 80 suspension under the same conditions. All experiments were performed following the recommendations given in the NIH Guide for the Care and Use of Laboratory Animals (8^{th} edition, 2010) and the Peruvian guide for mice care and management "INS, Guía de Manejo y Cuidado de Animales de Laboratorio: Ratón" (1^{th} edition, 2008). All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

Acute toxicity study in mice

Mice were treated in two phases according to Lorke 1983. In the first, p.o. doses of 10, 100 and 1000 mg/kg of crude extracts were administered to three groups, each with three animals. In the second, doses of 1600, 2900 and 5000 mg/kg were administered to groups, each of one animal. In both phases, mice were observed daily for a period of 7 days for mortality. Toxic effects and/or changes in behavioral patterns and physiological functions were also recorded using a data sheet for noting primary observations (Irwin test) as detailed by Roux et al. 2005.

Mice forced swimming test (FST)

The method used was that described by Porsolt et al. 1977. Mice were individually placed in a cylinder (25 cm height, 13 cm diameter) containing 10 cm water (22 ± 1 °C), from which they could not escape for a period of 6 min. All test sessions were recorded by a video camera located directly above the cylinders and tests were conducted between 9:00 and 13:00 h. Videotapes were later scored blind. The behavioral measurement scored from videotapes was

the duration of immobility during the last 4 min of the test period. A mouse was judged immobile when it ceased all active movements (i.e., struggling, swimming and/or jumping) and remained passively floating or making only the minimal movements necessary to maintain the nostrils above water. The extracts were assayed at the doses of 125, 250 and 500 and fluoxetine-HCl as a positive control at the dose of 30 mg/kg following a prior dose-response curve (**Table S1**).

Statistical analysis

One-way analyses of variance (ANOVA), followed by Dunnett post-hoc tests for multiple comparisons were carried out using GraphPad Prisma software 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Data are expressed as means ± S.E.M. Differences with $p<0.05$ between experimental groups were considered statistically significant.

Results and Discussion

Chemical analysis of the ethanolic extracts prepared from Peruvian Hypericum *species*

The analyses by TLC showed that the extracts were rich in flavonoids (**Fig. S1**). A principal band with the same R_f and chromogenic pattern of **3** was seen in the four extracts. Additionally, a blue fluorescent band with the same chromatographic pattern of phenolic acids was observed in the extracts, and was strongest in *H. andinum*. No band with the same chromatographic behavior as the flavonoid rutin or the reddish bands characteristics of naphthodianthrones were observed.

The initial HPLC-DAD analysis, using the method of Ganzera et al. 2002, reinforced the observations provided by TLC that the main secondary metabolites present in the extracts were flavonoids and related compounds (**Fig. S2**). The HPLC chromatograms of the four extracts contained peaks with UV spectra characteristic of flavonoids and phenolic acid derivatives (peaks eluting up to 16 min of t_R). In the lipophilic region (peaks eluting after 19 min of t_R), some minor peaks with characteristic three peaked UV spectra (maxima of 230, 290 and 340 nm) and other peaks with strong absorbance peaks at 220 and 300 nm were observed. No peak with UV spectra characteristic of naphthodianthrones was observed. The

absence of naphthodianthrones in species of *Hypericum* from sections *Brathys* and *Trigynobrathys* has been previous cited (Crockett and Robson 2011)

Further efforts were made to characterize the extracts qualitatively and quantitatively by HPLC, following the method of Tatsis et al. 2007, associated to UPLC-DAD/Q-TOF-MS (**Fig. S4** and **Fig. S5**). The overlaid HPLC chromatograms and the principal components detected in the extracts of these *Hypericum* species are shown in **Fig. 1**. All four extracts were rich in flavonoids, especially **3** (**Table 2**) which occurred in the highest concentration in *H. andinum* (11.94%), and the lowest in *H. laricifolium* (7.03%). Compound **5** was also observed in concentrations ranging from 0.55 to 0.73%. A considerable concentrations of **3** was observed in *H. silenoides* (2.90%). Compounds **1** and **2** and were not detected in the four samples analyzed, even though **1** is described as occurring in several Brazilian *Hypericum* species of section *Trigynobrathys* (Nunes et al. 2010; Barros et al. 2013), and **2** has been described in *H. brasiliense* and a Mexican accession of *H. silenoides* (Abreu et al. 2004; García-de la Cruz et al. 2013). Compound **3** was previously reported at a concentration of 17.17% in a methanolic extract from flowers of *H. andinum*, which was the highest concentration found among eight Brazilian and two Peruvian *Hypericum* species extracts (Barros et al. 2013). Similarly, the extract of *H. andinum* displayed the highest concentrations of **3** of the four species, and these four extracts showed higher concentrations of **3** when compared to previously published data for Brazilian *Hypericum* species, except *H. myrianthum* (Nunes et al. 2010; Barros et al. 2013). The *Hypericum* species included in this study were collected in several Peruvian Páramos habitats at a high altitude, exceeding 3,000 m above the sea level. The flavonoid content of some *Hypericum* species has been previously noted to positively correlate with altitude (Umek et al. 1999), and our observation of high content of **3** in Peruvian *Hypericum* species strengthens this correlation. The Peruvian Páramo habitats are exposed to high levels of UV radiation that could led to the induction of flavonoid biosynthesis (Casati and Walbot 2005; Jaakola and Hohtola 2010).

In the lipophilic region of the chromatogram, some peaks with characteristics corresponding to those of dimeric acylphloroglucinols were detected. Compounds **6**, **7** and **9** were present in the four samples in varying amounts. The concentration of **6** was highest in *H. silenoides* (0.86 %), while its concentration ranged from 0.06 to 0.11% in the other species. In general, the concentration of **7** was similarly in each of the four samples, while concentrations of **9**

ranged from 0.08 to 0.17%. Compounds **8** and **10** were only detected in *H. laricifolium* in concentrations of 0.04 and 0.03%, respectively. These results are in concordance with those of a previous study, in which **6**, **7** and **9** were isolates from *n*-hexane extracts of the same accessions of the four *Hypericum* species, while **8** and **10** were isolated from *H. laricifolium*; the chemotaxonomic implications of these findings are also discussed elsewhere (Ccana-Ccapatinta et al. 2013). Even though these compounds appeared as a single band on TLC using several mobile phases and each displayed clear signals in the ^{1}H and ^{13}C NMR spectra, each isolated dimeric acylphloroglucinol displayed a minor peak (named **6a**, **7a**, **8a**, **9a** and **10a**) in the HPLC chromatograms (**Fig. S3**). The identity of these minor components is currently under investigation.

Assessing the antidepressant like activity of the ethanolic extracts prepared from Peruvian Hypericum *species*

The ethnopharmacological uses of some Peruvian *Hypericum* species in northern Peru have recently been published (Bussmann and Glenn 2010; Bussmann et al. 2010). Of the four species surveyed here, only extracts of *H. silenoides* has been reported to be taken orally as an antimicrobial preparation (Bussmann et al. 2010), and to the best of our knowledge no ethnopharmacological records exist for the use if these species as remedies for central nervous system related illnesses. Therefore, an acute toxicity test was carried out following the method of Lorke 1983 (**Table S2**). The extracts of *Hypericum* species administered p.o. did not induce mortality up to a dose of 5000 mg/kg in mice, up to 7 days after administration. The tested animals did not present any symptoms of toxicity, however, the mice that received *H. brevistylum* at 2900 and especially 5000 mg/kg showed signs of nervous system sedation, a loss of balance, and finally akinesia. Similar results have been previously observed with alcoholic extracts of other *Hypericum* species (Rieli Mendes et al. 2002; Sánchez-Mateo et al. 2002).

The results of the FST, following the method of Porsolt et al. 1977, are summarized in **Table 3**. In this test, immobility postures are observed and assumed to be a depressive-like parameter. All *Hypericum* extracts at different doses significantly shortened the immobility time in the FST in comparison to the control group. The ethanolic extract of *H. brevistylum* (500 mg/kg), in this regard, the most effective, with an activity value (58.47 %) close to that

observed for the positive control, fluoxetine-HCl at 30 mg/kg. Compared with the control group, significant differences were seen between *H. andinum* 125 ($p<0.05$), *H. andinum* 250 ($p<0.01$), *H. andinum* 500 ($p<0.01$), *H. brevistylum* 250 ($p<0.001$), *H. laricifolium* 250 ($p<0.05$) and *H. silenoides* 500 mg/kg ($p<0.001$) as assessed by ANOVA among the fourteen groups. U-shaped dose-response curves were observed in the groups that received extracts of *H. brevistylum* and *H. laricifolium*. A previous study showed that an alcoholic extract of *H. perforatum* at doses of 125, 250, 500 and 1000 mg/kg significantly reduce the immobility time in the rat FST, displaying a U-shaped dose-response curve (Butterweck et al. 1997).

Dimeric acylphloroglucinols were detected in low concentration in the Peruvian *Hypericum* extracts, while naphthodianthrones were absent. Compound **9** was cited to reduce the immobility time in the FST at 10 mg/kg, p.o. by Stein et al. 2012, but the concentration of this compound in the Peruvian *Hypericum* extracts are low to fully account for the overall activity observed. Compounds **6**, **7**, **8** and **10** are structurally related to **9** and may contribute to the effect observed, but their concentrations are also lower than 0.1% in the extracts. Further studies are needed to determine the overall contribution of dimeric acylphloroglucinols in the antidepressant-like activity of the crude extracts.

In contrast to dimeric acylphloroglucinols, some flavonoids were abundant in the extracts. Compounds **5** and especially **3** were identified as primary secondary metabolites shared among these extracts. Fractions and the isolated compound **3**, from *H. perforatum* and *H. caprifoliatum*, reduced the immobility time in the rat FST at 0.3 and 1.2 mg/kg, p.o. (Butterweck et al. 1997; Butterweck et al. 2000), and in the mice FST at 10 and 20 mg/kg, i.p. (Haas et al. 2010). Since the extracts of Peruvian *Hypericum* species are rich in 3, the antidepressant-like activity observed could be attributed mainly to this flavonoid and, to a lesser extent, to the dimeric acylphloroglucinols. Even though it has demonstrated selective MAO-A inhibitory activity in the nanomolar range, **5** has failed to reduce the immobility time in the FST, and, therefore, its antidepressant-like behavioral effects are considered controversial (Butterweck et al. 2000; Chimenti et al. 2006). Further studies are ongoing in our laboratory to examine the contribution of each main component to the overall effects observed this study. In addition, the issues of possible synergism and/or antagonism must be addressed.

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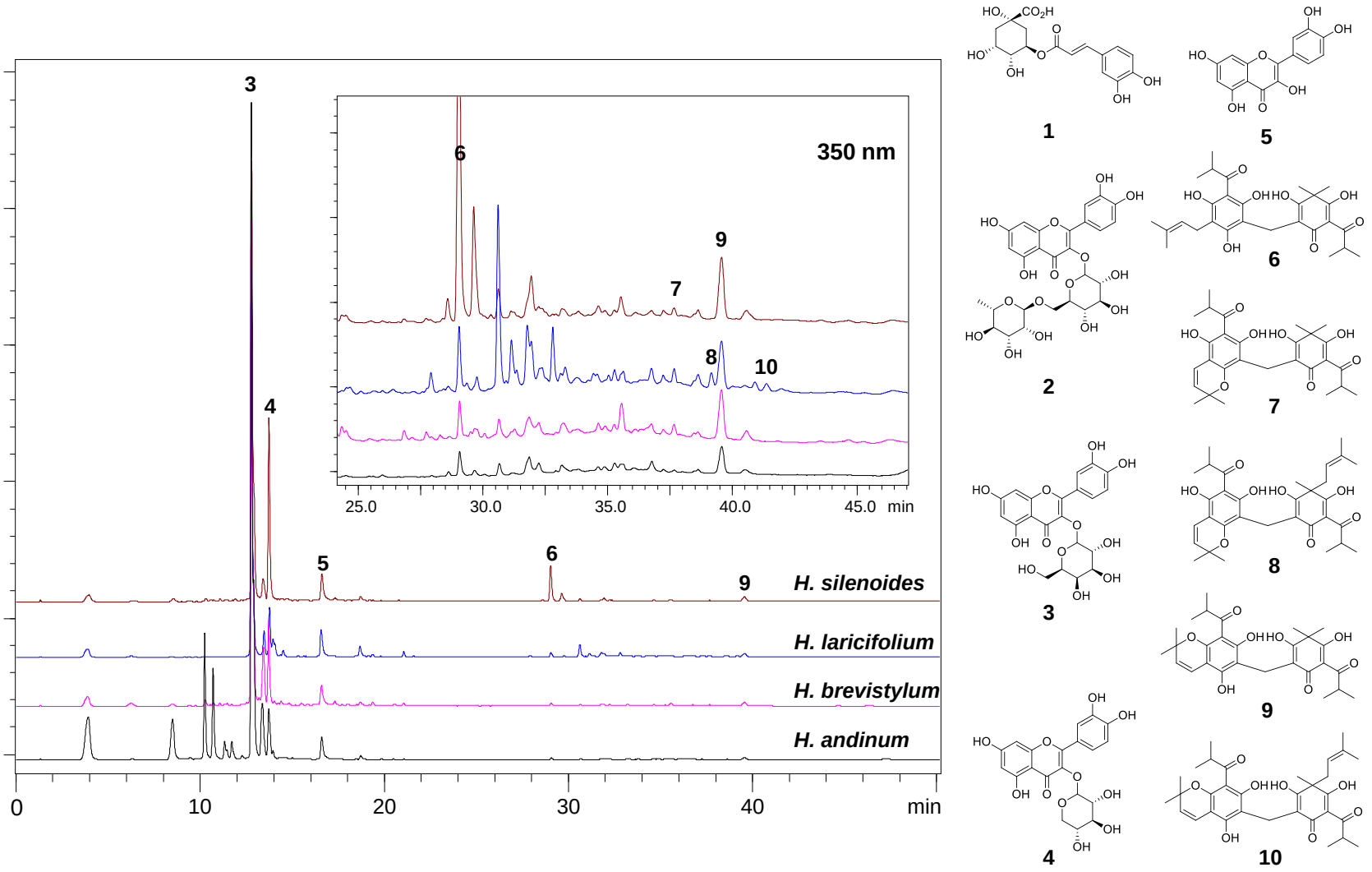

Fig. 1 HPLC chromatogram overlaid and principal components identified in the ethanolic extracts of Peruvian *Hypericum* species: chlorogenic acid (**1**), rutin (**2**), hyperoside (**3**), guaijaverin (**4**), quercetin (**5**), uliginosin A (**6**), isouliginosin B (**7**), isohyperbrasilol B (**8**), uliginosin B (**9**), hyperbrasilol B (**10**)

Table 1 Collection localities of Peruvian *Hypericum* species

Species	Collection locality	Elevation[a]	Collection Date	Voucher number
H. andinum	Amparaes, Cuzco, Peru	3432	May 2008	Ccana-Ccapatinta 06
H. brevistylum	Paqchaq, Cuzco, Peru	3573	March 2008	Ccana-Ccapatinta 04
H. laricifolium	Cumbemayo, Cajamarca, Peru	3450	November 2009	Ccana-Ccapatinta 08
H. silenoides	Pumahuanca, Cuzco, Peru	3189	March 2008	Ccana-Ccapatinta 02

[a]meters above sea level

Table 2 UV, MS and content (%) of identified compounds in the ethanolic extracts of *Hypericum* species

	HPLC t_R (min)	UPLC t_R (min)	UV λmax (nm)	ESI $[M+H]^+$ (m/z)	Characteristic Ions	*H. andinum*	*H. brevistylum*	*H. laricifolium*	*H. silenoides*
1	7.51	1.92	217, 325	355	377$[M + Na]^+$, 163	n.d.	n.d	n.d	n.d
2	12.52	2.67	255, 353	611	633$[M + Na]^+$, 465, 303	n.d.	n.d	n.d	n.d
3	12.77	2.69	255, 353	465	487$[M + Na]^+$, 303	11.94	8.83	7.03	8.11
4	13.41	2.90	255, 353	435	457$[M + Na]^+$, 303	+	+	+	2.90
5	16.59	3.66	255, 354	303	-	0.63	0.55	0.73	0.71
6	29.05	8.27	227, 299, 347	501	-	0.06	0.07	0.11	0.86
7	37.66	9.51	231, 268,	499	-	0.02	0.03	0.04	0.02
8	39.09	9.81	269	553	-	n.d.	n.d	0.04	n.d
9	39.56	9.85	229, 269	499	-	0.08	0.14	0.13	0.17
10	40.81	10.10	230, 269	553	-	n.d.	n.d	0.03	n.d

n.d., not detected; +, presence, not quantified, overlapping peaks

Table 3 Results of the forced swimming test in mice of the ethanolic extracts of *Hypericum* species

Treatment	Dose (p.o.) (mg/kg)	N	Duration of Immobility (Mean ± S.E.M.) (s)	Variation (%)
Control	-	10	202.0 ± 7.31	-
Fluoxetine	30	10	91.50 ± 19.25***	-54.70
H. andinum	125	10	131.5 ± 18.68*	-35.15
H. andinum	250	9	115.0 ± 9.20**	-43.07
H. andinum	500	10	120.5 ± 12.21**	-40.35
H. brevistylum	125	10	168.0 ± 10.65	-16.83
H. brevistylum	250	9	83.89 ± 15.76***	-58.47
H. brevistylum	500	9	156.1 ± 15.76	-22.77
H. laricifolium	125	10	158.0 ± 15.11	-21.78
H. laricifolium	250	10	139.5 ± 19.08*	-30.94
H. laricifolium	500	9	175.6 ± 12.48	-13.06
H. silenoides	125	10	150.5 ± 8.865	-25.49
H. silenoides	250	9	188.9 ± 11.78	-6.49
H. silenoides	500	9	93.89 ± 21.09***	-53.52

*p<0.05, **p<0.01, ***p<0.001, compared with control group (ANOVA followed by Dunnett´s multiple comparison test). N, number of animals per group

Table 4 Presence and content (%) of major compounds in hydroalcoholic crude extracts of some *Hypericum* species with reported antidepressant like activity in the murine forced swimming test

Species*	1	2	3	4	5	hypericin	hyperforin	Reference
H. brasiliense[a]	+	n.d.	+	+	+	n.d.	n.d.	Perazzo et al. 2008
H. enshiense[b]	1.0	0.2	0.8	n.r.	1.0	0.3	n.d.	Wang et al. 2010
H. grandifolium[c]	n.r.	n.d.	0.7	n.r.	3.2	n.d.	< 0.5	Bonkanka et al. 2008
H. montbretti[d]	<0.01	0.15	<0.01	n.r.	<0.01	<0.01	<0.01	Can et al. 2011
H. origanifolium[e]	0.23	0.21	<0.01	n.r.	<0.01	<0.01	0.01	Yaşar et al. 2013
H. perforatum[g]	n.r.	<4.0; 2.0>	<3.0; 1.5>	n.r.	<0.5	>0.24	<4.0; 2.0>	Kurth and Spreemann 1998
H. silenoides[i]	+	+	n.r.	n.r.	+	n.d.	+	García-de la Cruz et al. 2013

*Hydroalcoholic extracts of *H. calycinum* (Öztürk et al. 1996) *H. caprifoliatum* (Daudt et al. 2000), *H. canariense*, *H. glandulosum*, *H. reflexum* (Sánchez-Mateo et al. 2002), *H. cordatum* (Rieli Mendes et al. 2002), *H. laricifolium* (Laines Lozano 2010) and *H. scabrum* (Eslami et al. 2011) were also active in the FST.

[a]Ethanolic extract previously extracted with hexane. Reported to have 8.5 % of flavonoids calculated in quercetin.

[b]Hydroalcoholic extract with 80% ethanol. Pseudohypericin constituted 0.5 % of the extract.

[c]Methanolic extract also surveyed by Sánchez-Mateo et al. 2002. Quercitrin constituted 0.6 % of the extract.

[d]Methanolic extract. Quercitrin and isoquercitrin constituted 0.07 and <0.01% of the extract.

[e]Hydroalcoholic extract with 50% ethanol. Quercitrin and isoquercitrin constituted 0.03 and <0.01% of the extract.

[g]Ranges for flavonoids, hypericin and hyperforin constituents of HyperiFin™ commercial extract. Isoquercitrin, approximately 1%; quercitrin, not more than 0.5%; biapigenin, approximately 0.3%.

[i]Aqueous extract.

n.d., not detected. n.r., not reported. +, presence

Assessing the phytochemical profiles and antidepressant-like activity of four Peruvian *Hypericum* species using the murine forced swimming test

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Supplementary information:

Table S1 Results of the forced swimming test in mice of different doses of fluoxetine-HCl

Treatment	Dose (p.o.) (mg/kg)	N	Duration of Immobility (Mean ± S.E.M.) (s)	Variation (%)
Control	-	6	160.0 ± 4.08	-
Fluoxetine	20	6	120.0 ± 21.64	-25.00
Fluoxetine	30	5	68.0 ± 25.13**	-57.50
Fluoxetine	40	6	107.5 ± 15.64	-32.81

***p*<0.01, compared with control (ANOVA followed by Dunnett´s multiple comparison test). N, number of animals per group

Table S2 Results of the acute toxicity study in mice of the ethanolic extracts of *Hypericum* species

Species	Phase I dose (mg/kg)			Phase II dose (mg/kg)			DL_{50} (mg/kg)
	10	**100**	**1000**	**1600**	**2900**	**5000**	
H. andinum	0/3[a]	0/3	0/3	0/1	0/1	0/1	>5000
H. brevistylum	0/3	0/3	0/3	0/1	0/1	0/1	>5000
H. laricifolium	0/3	0/3	0/3	0/1	0/1	0/1	>5000
H. silenoides	0/3	0/3	0/3	0/1	0/1	0/1	>5000

[a]Number of animals dead/number of animals used

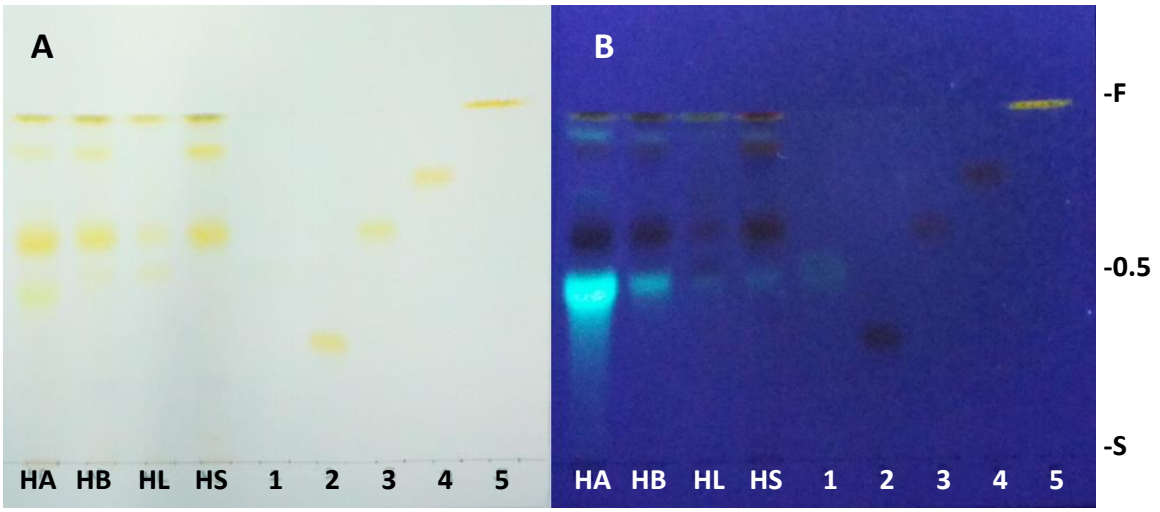

Fig. S1 TLC of flavonoids and phenolic acids in the ethanolic extracts of Peruvian *Hypericum* species. Separation on aluminum sheets precoated with silica gel 60 F_{254} with ethyl acetate/formic acid/acetic acid/water - 100:11:11:26 (v/v) as mobile phase. (A) visible light; (B) UV light at 365 nm; HA, *H. andinum*; HB, *H. brevistylum*; HL, *H. laricifolium*; HS, *H. silenoides*; **1**, chlorogenic acid; **2**, rutin; **3**, hyperoside; **4**, guaijaverin; **5**, quercetin

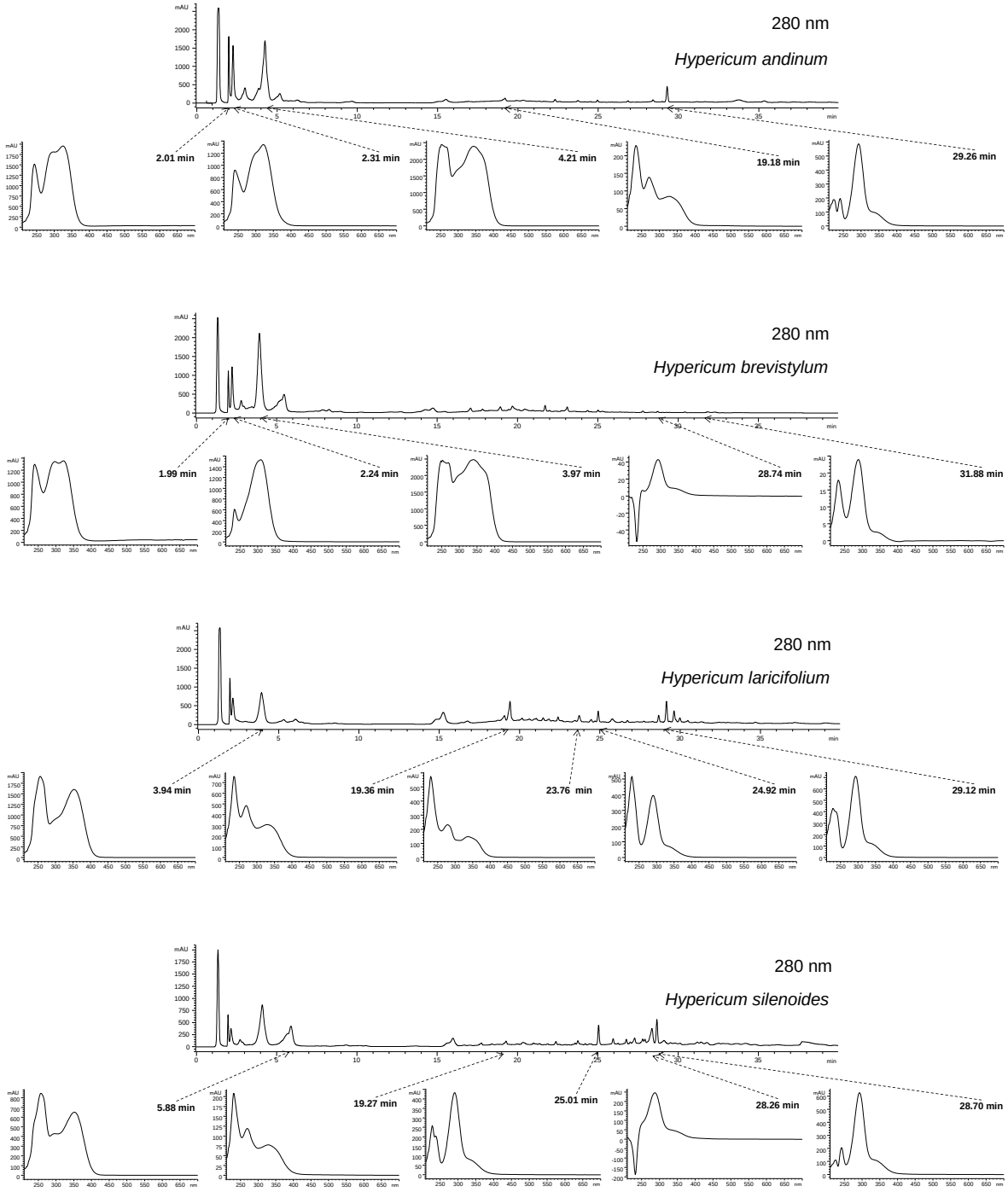

Fig. S2 HPLC-DAD chromatogram and UV spectra of principal and some minor components in the ethanolic extract of *H. andinum*, *H. brevistylum*, *H. laricifolium* and *H. silenoides* (following the method described in Ganzera et al. 2002)

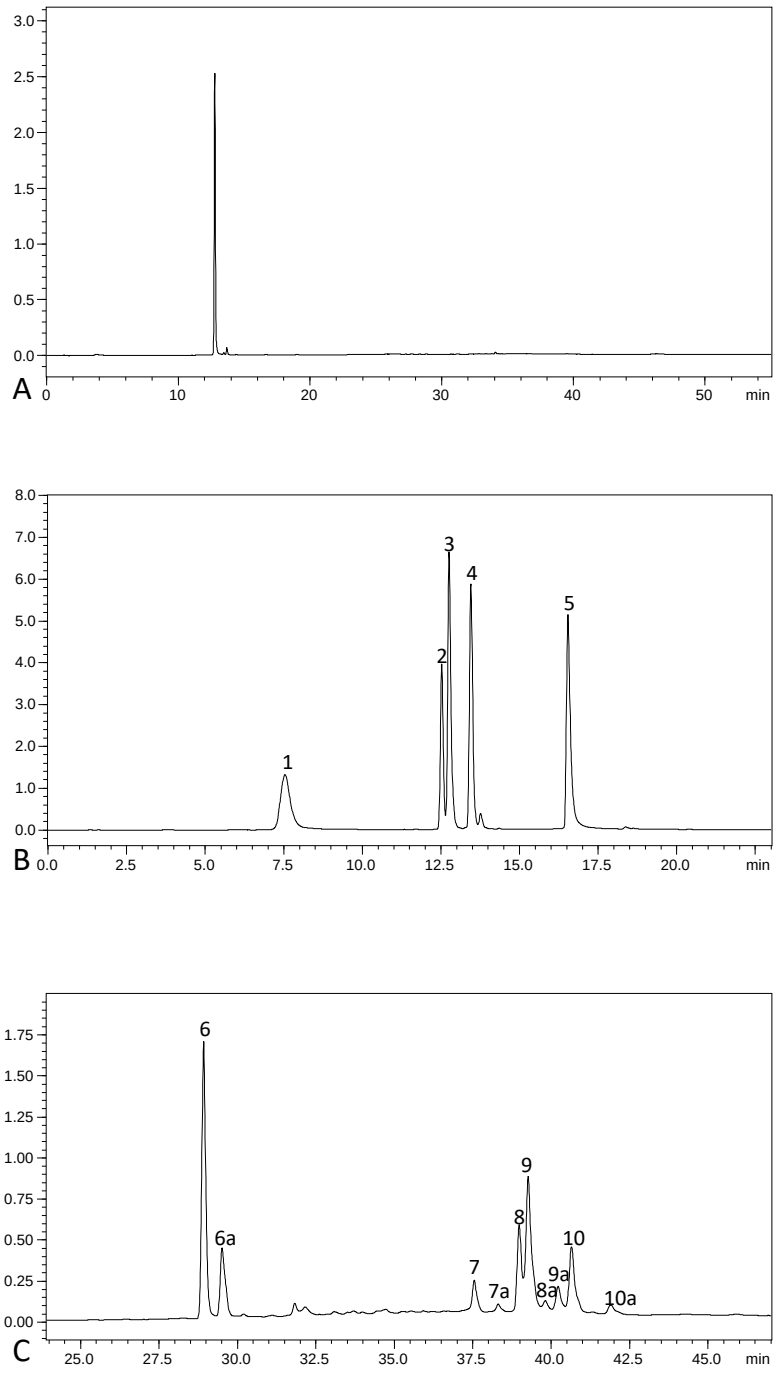

Fig. S3 HPLC chromatograms of reference substances**.** (A) Hyperoside isolated from *H. andinum*. (B) Mixture of flavonoids and a phenolic acid: **1**, chlorogenic acid; **2**, rutin; **3**, hyperoside; **4**, guaijaverin; **5**, quercetin. (C) Mixture of dimeric acylphloroglucinol: **6**, uliginosin A; **7**, isouliginosin B; **8**, isohyperbrasilol B; **9**, uliginosin B; **10**, hyperbrasilol B. Each reference substance displayed a minor peak named as **6a**, **7a**, **8a**, **9a** and **10a** (following the method described in Tatsis et al. 2007)

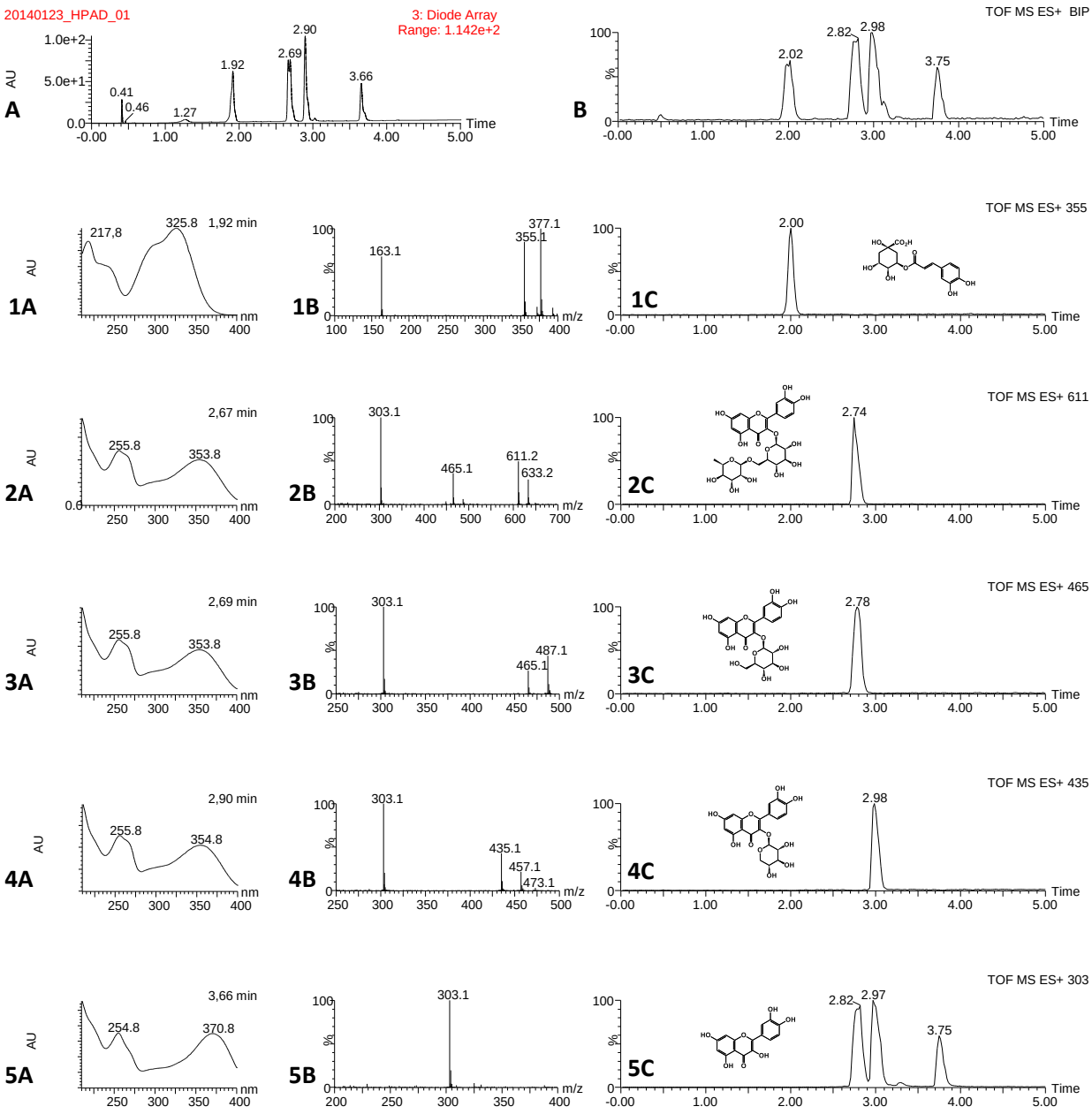

Fig. S4 UPLC-DAD/Q-TOF-MS chromatogram of reference substances: (A) UPLC-DAD chromatogram; (B) base peak ion (BIP) chromatogram in positive mode (*m/z* range 50-1000); (1A - 5A) UV spectra of flavonoids and a phenolic acid, **1** (chlorogenic acid), **2** (rutin), **3** (hyperoside), **4** (guaijaverin) and **5** (quercetin); (1B - 5B) MS spectra of flavonoids and a phenolic acid in positive mode; (1C - 5C) reconstructed BIP chromatogram at *m/z* values of 355, 611, 465, 435 and 303

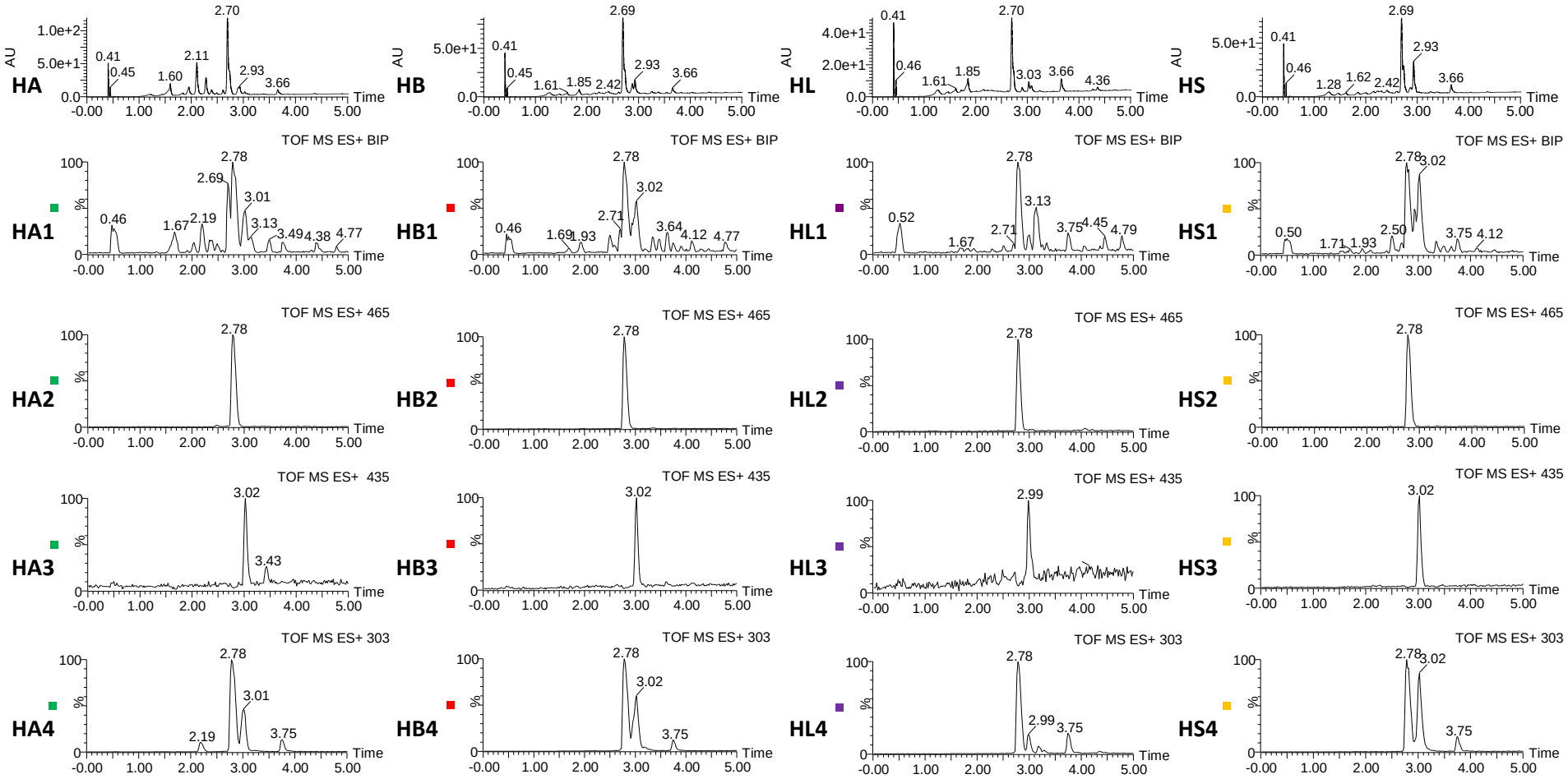

Fig. S5 UPLC-DAD/Q-TOF-MS chromatograms (phenolic acids and flavonoids region) of ethanolic extracts of Peruvian *Hypericum* species: (HA, HB, HL and HS) UPLC-DAD chromatograms of *H. andinum* (HA) , *H. brevistylum* (HB), *H. laricifolium* (HL) and *H. silenoides* (HS); (HA1, HB1, HL1 and HS1) base ion peak (BIP) chromatograms in positive mode (*m*/*z* range 50-1000); reconstructed BIP chromatograms at *m*/*z* values of 465 (HA2, HB2, HL2 and HS2), 435 (HA3, HB3, HL3 and HS3) and 303 (HA4, HB4, HL4 and HS4). Notice: entire chromatogram last 15 min, nevertheless peaks corresponding to dimeric acylphloroglucinols were of low intensity

CAPÍTULO IV

"Dimeric acylphloroglucinol rich *n*-hexane extracts of Peruvian *Hypericum* species with antichemotactic activity: fingerprint and chemometrics"

Manuscrito em preparação

Dimeric acylphloroglucinol rich *n*-hexane extracts of Peruvian *Hypericum* species with antichemotactic activity: fingerprint and chemometrics

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The genus *Hypericum* (Hypericaceae) is a prolific source of numerous molecules that include principally flavonoids, xanthones, naphthodianthrones, and acylphloroglucinol derivatives (Crockett 2012). The presence of mono-, bi-, and tricyclic isoprenylated acylphloroglucinols, as well as, mono- and dimeric acylphloroglucinols in lipophilic fractions from representatives of *Hypericum* is frequently reported, providing evidence that *Hypericum* displays a specialized chemical diversity of acylphloroglucinol derivatives (Pal Singh and Bharate 2006; Crockett 2012; Stojanović et al. 2013).

Hyperforin is certainly the most well-known acylphloroglucinol of the genus *Hypericum*. It is the main acylphloroglucinol of *H. perforatum* and also regarded as one of the main active principles responsible for the recognized antidepressant activity of St John's wort phytochemical preparations. Among many other bioactivities (Medina et al. 2006), hyperforin exhibited potent anti-inflammatory effect on a UV erythema test associated to its *in vitro* and *ex vivo* antioxidant activity in skin and skin cells (Meinke et al. 2012), to its ability to suppress the *in vivo* and *in vitro* prostaglandin (PG)E_2 biosynthesis (Koeberle et al. 2011), and to block other pro-inflammatory functions of leukocytes such as chemotaxis and chemoinvasion (Dell'Aica et al. 2007).

Brathys and *Trigynobrathys* (88 and 59 species respectively) are the two largest sections of the genus *Hypericum* that are principally distributed in Central and South America and, in a less extent, North America, Australia and Asia (Robson 2012). Of the more than 100 South American species of *Hypericum* almost 65 are endemic to the Páramo, a high-altitude grassland ecosystem characterized by vegetation composed mainly of giant rosette plants, shrubs and grasses (Crockett et al. 2010; Nürk et al. 2013).

Differently to the polyisoprenylated acylphloroglucinol hyperforin, species of *Hypericum* from sections *Brathys* and *Trigynobrathys* are described to produce mainly dimeric acylphloroglucinol structures consisting of a filicinic acid and a phloroglucinol moieties linked by a methylene bridge (Barros et al. 2013; Ccana-Ccapatinta et al. 2013). To date, 31 unique structures of dimeric acylphloroglucinols are described, which have shown to display a wide array of bioactivities as antimicrobials, antidepressants, analgesics, and anti-inflammatories (Ccana-Ccapatinta et al. 2013).

Fractions rich in dimeric acylphloroglucinols saroaspidin A, uliginosin A and hyperbrasilol A from *H. gentianoides* were shown to potently inhibit the *Escherichia coli* lipopolysaccharide (LPS)-induced prostaglandin E_2 (PGE_2) and nitric oxide (NO) production in RAW 264.7 mouse macrophages (Huang et al. 2011). Likewise, lipophilic extracts from three southern Brazilian *Hypericum* species, rich in dimeric acylphloroglucinols uliginosin B, japonicin A and hyperbrasilol B, inhibited markedly the *in vitro* LPS-induced chemotaxis on rat polymorphonuclear neutrophils (PMNs) (Barros et al. 2013).

Since *Hypericum* species from section *Brathys* and *Trigynobrathys* are potential alternative sources of anti-inflammatories, the present study was aimed to evaluate the ability of lipophilic extracts from six Páramo Peruvian *Hypericum* species to inhibit the *in vitro* LPS-induced chemotaxis on rat PMNs. These extracts are hypothesized to be rich in dimeric acylphloroglucinol structures, therefore their chemical composition were characterized by a HPLC-DAD fingerprint method, associated with LC-ESI-MS, UPLC-Q-TOF-MS, principal component analysis (PCA) and hierarchical clustering analysis (HCA).

Material and methods

Plant material

Hypericum aciculare Kunth (HACI), *H. andinum* Gleason (HAND), *H. decandrum* Turcz (HDEC), *H. laricifolium* Juss (HLAR) (Shrubs, section *Brathys*), *H. brevistylum* Choisy (HBRE) and *H. silenoides* Juss (HSIL) (herbs, section *Trigynobrathys*) are species that occur in high altitude Páramo habitats. Aerial parts of these plants were collected in some regions of Peru (**Table 1**) and identified by Botanist MsC. Washington H. Galiano Sánchez. Collection authorization 0147-2010-AG-DGFFS-DGEFFS was issued by the Dirección de Gestión Forestal y de Fauna Silvestre and Dirección General Forestal y de Fauna Silvestre of the Republic of Peru. Voucher specimens were deposited in the Herbarium of the Federal University of Rio Grande do Sul (ICN), Brazil and in the Herbarium of the Universidad Nacional de San Antonio Abad del Cusco (CUZ), Peru.

Preparation of plant extracts and reference substances

Air dried and powdered vegetal material (10g) was successively extracted with *n*-hexane with the aid of an ultrasonic bath (5 times of 15 min, plant-solvent ratio 1:10). The extracts were evaporated to dryness under reduced pressure and then treated with cold acetone to obtain respective acetone soluble fractions (3.20, 2.48, 2.92, 2.57, 4.24 and 4.52% for HACI, HAND, HBRE, HDEC, HLAR and HSIL, respectively). The extracts were stored under refrigeration and dissolved in HPLC grade methanol (2000, 1000, 500, 250 and 100 µg/mL) and filtered (0.22 µm pore size) once required.

Uliginosin A (**1**), isouliginosin B (**2**), isohyperbrasilol B (**3**), uliginosin B (**4**) and hyperbrasilol B (**5**) were isolated from some Peruvian *Hypericum* spp. by chromatographic procedures and identified after the analysis of their 1D and, 2D NMR spectra as described elsewhere (Ccana-Ccapatinta et al. 2013). Further purification attempts were carried out by centrifugal thin-layer chromatography (Chromatotron^{TM}, San Bruno, CA, USA) on 1mm silica gel 60 F_{254} (Merck, Darmstadt, Germany) plates using several mobile phase mixtures described elsewhere (Rocha et al. 1995). The purity of each isolated compound were proved to be 75, 70, 91, 73 and 90%, respectively, by HPLC analysis.

High-performance liquid chromatography (HPLC)

HPLC-DAD separations were performed on a Waters Nova-Pack C18 column (4 μm, 3.9 mm x 150 mm) adapted to a guard column Waters Nova-Pack C18 60Å (Waters, Milford, MA, USA) using a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with a UV/Vis/DAD SPD-M20A system detector. The mobile phases consisted of a mixture of water (A) and an 8:2 mixture of acetonitrile and methanol (B), both acidified with 0.1% formic acid. Gradient elution was performed as follows: linear gradient from 75% B to 100% B over 10 min, held at 100% B for 10 min, followed by a re-equilibration for 5 min. All separations were performed at 25°C and a flow rate of 1.0 mL/min. Scan wavelength was set from 190 to 400 nm and detection wavelength at 350 nm. An aliquot of 20 μL of extract per sample (1000 μg/mL) was injected to obtain the fingerprint chromatograms and for quantifying purposes. Comparison of retention times (t_R) and UV spectra of standard injection peaks (isolated substances) against sample peaks was used to assign peaks identity. Spiking of samples with standard compounds was employed to confirm assignments.

Fingerprinting method validation

The precision of the chromatographic method was assessed by performing nine consecutive injections of the same concentration of HLAR (1000 μg/mL). The repeatability of the extraction procedure and subsequent analysis was performed by repeating the extraction and HPLC analysis procedure by six times on the same sample of HLAR (1000 μg/mL). The intra-day reproducibility was performed by three repeated analyses at four concentration points of HLAR (2000, 1000, 500 and 100 μg/mL). The inter-day reproducibility was also evaluated by analyzing a sample of HLAR (1000, 500 μg/mL) on three consecutive days. The relative standard deviation (RSD) of retention times (t_R) and peak concentration (PC) of three selected peaks were used to determine the method precision, repeatability, intra- and inter-day reproducibility.

The relative concentration of dimeric acylphloroglucinols in the *n*-hexane extracts were quantified using a calibration curve prepared with **5** dissolved in MeOH with six data points covering the concentration range of 0.9–450 μg/mL. These solutions were injected in

triplicate, on three different days. The calibration curve was obtained by plotting the peak area signals as a function of concentration.

Chemometric analyses

The HPLC-DAD fingerprint chromatograms and UV spectra of major peaks were submitted to principal component analysis (PCA) and hierarchical clustering analysis (HCA) performed with the MINITAB® 15 software (State College, PA, USA). HCA: Agglomerative hierarchical method and Ward's linkage method were employed to determine the number and distance between clusters, respectively and Euclidean distance for their amalgamation. PCA: the number of components was determined from Kaiser's eigenvalue greater than 1.0 rule employing the correlation matrix.

Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS)

Mass detection was carried out on an Agilent 1260 infinity LC-ESI-MS instrument equipped with an Agilent 6120B series mass detector (Agilent Technologies, Palo Alto, CA, USA). The analytical column, mobile phase composition and gradient elution were the same as described in the HPLC section. An aliquot of 10 µL of extract per sample (500 µg/mL) was injected. The mass spectrometer was operated with an electrospray ionization source in positive (ESI+) and negative (ESI-) mode. Detection was initially carried out from *m/z* = 100 to 1000 and further optimized from *m/z* = 400 to 700. The following parameters were set to spray chamber: capillary voltage 3.0 kV, drying gas flow 10.0 L/min, nebulizer pressure 50 psig, drying gas temperature 350 °C and fragmentor voltage kept at 100V.

UPLC -Q-TOF-MS

MS/MS spectra of principal peaks were characterized by UPLC-Q-TOF-MS using a Waters Acquity UPLC system coupled to a Waters Q-TOF Premier mass spectrometer (Waters, MA, Milford, USA). Separations were performed on a Hypersil Gold C18 column (1.9 µm, 2.1 mm x 100 mm). The mobile phases consisted of a mixture of water (A) and an 8:2 mixture of acetonitrile and methanol (B), both acidulated with 0.1% formic acid. Gradient elution was performed as follows: linear gradient from 75% B to 100% B over 2.7 min, held at 100% B

for 2.9 min, followed by a re-equilibration for 1.4 min. All separations were performed at 30°C and a column flow rate was 0.49 mL/min. An aliquot of 2.5 µL of extract (250 µg/mL) per sample was injected. Mass detection was carried out in positive mode from *m/z* = 50 to 1000 and data were processed using MassLynx V4.1 software (Waters, MA, Milford, USA). Nitrogen and Argon were used as nebulizer and collision gas, respectively. Other detection condition were as follows: ESI capillary voltage, +3.0kV; source and desolvation temperatures, 120 and 250°C, respectively; desolvation and cone gas flows, 350 and 70 L/h, respectively; sample cone voltage 25V ; collision energy 35 eV.

Antichemotactic activity assay

The antichemotactic activity assays of the *n*-hexane extracts on rat PMNs were performed according to the method described by Suyenaga et al. (2011). Briefly, stock solutions (1000 µg/mL) of the extracts were prepared with Hanks buffer and Tween 80 (maximum concentration = 10%). The stock solutions were diluted in rat leukocyte solution (leukocyte density of about 1.5×10^6 cells/mL) to obtain suspensions with extract concentrations of 0.01 to 10.0 µg/mL that were incubated at 37°C for 30 min. Then, the leukocyte/extract suspensions were added to the upper wells of a modified Boyden chamber, separated from the chemotactic stimulant (LPS from *Escherichia coli*) present in the lower compartment by an 8.0 µm nitrocellulose filter (Millipore, Billerica, MA, USA). Positive (lower wells containing LPS) and negative controls (lower wells containing medium only) were conducted in parallel with experimental groups. The Boyden chambers were incubated at 37°C in a humidified atmosphere for 1 h and, thereafter, the filters were removed and stained. The leucocytes migration was measured using a microscope Alphaphot-2 YS2 (Nikon, Tokyo, Japan). The mean value of migration corresponds to the micrometer distance from the top of the filter to the farthest plane of focus still containing two cells in five microscopic fields. All experiments were carried out in triplicate. A stock solution of uliginosin B (1000 µM) was also prepared and diluted to concentrations of 0.01 to 100 µM. Indomethacin (28 µM) was used as reference drug.

Statistical analysis

Differences between the control and the treatments were statistically analyzed by one-way analysis of variance (ANOVA), followed by Dunnett post-hoc test for multiple comparisons. Data is expressed as means ± S.E.M. Differences with $p<0.05$ between experimental groups were considered statistically significant. Regression analysis was used to estimate the IC_{50} value for the inhibitory activity of uliginosin B using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

Results and discussion

Fingerprinting method validation

The precision of the method was determined by nine consecutive injections of HLAR. The RSDs of t_R and PC of selected peaks were below 1.9% and 5.6%, respectively. The repeatability test was performed with six independently prepared sample solutions of HLAR. The RSDs of t_R and PC were below 2.0% and 7.4%, respectively. The intraday reproducibility was performed by analyzing three repeated analyses at four concentration points of HLAR and The RSDs of t_R and PC percentages were below 1.2% and 4.2%, respectively. The inter-day reproducibility was also evaluated and the results showed acceptable RSD values (below 3.7% and 8.7%). Despite the complex nature of the sample extracts, these results indicate that the fingerprinting method is valid and applicable.

For the quantification of known dimeric acylphloroglucinols (and peaks with dimeric acylphloroglucinol pattern) in the *n*-hexane extracts, a calibration curve was constructed with compound **5** (Y = 4363.13 X - 14204.7) as external standard, displaying a good linearity (r^2 = 0.9999) in the range of concentrations analyzed. The limit of detection and limit of quantification were 0.68 and 2.05 µg/mL, respectively.

Dimeric acylphloroglucinols reference substances and its M + 14 higher homologues

Five dimeric acylphloroglucinol previously isolated from four Peruvian *Hypericum* species and identified after their respective 1D and 2D NMR spectra (Ccana-Ccapatinta et al. 2013), were used to assign peak identity on the fingerprint chromatograms of the *n*-hexane extracts from *Hypericum* species included in the present study. However, when these samples were

individually submitted to the HPLC fingerprint analysis each of them displayed a principal (**1 - 5**) and a minor peak (**1a - 5a**). The reference substances showed a single spot on TLC under several mobile phase systems and displayed clear signals in their ^{1}H and ^{13}C NMR spectra (Ccana-Ccapatinta et al. 2013) that could not reveal and identify the nature of these minor peaks, even though their concentrations in some samples were shown to be almost a quarter of the main peak (21, 15, 3, 25, and 8% for **1a**, **2a**, **3a**, **4a** and **5a**, respectively, by HPLC analysis), potentially identifiable by NMR spectroscopy. These observations, in addition to the fact that the UV spectra profile and the λ_{max} of both pair peaks were almost identical (determined by HLPC-DAD), and a difference of 14 mass units between both pair peaks were observed (determined by LC-ESI-MS), suggested a close structural similarity of the two pair peaks observed in each sample (**Fig. 1**).

The mayor peaks with *m/z* of 501 (t_R 3.25 min), 499 (t_R 15.26 min), 553 (t_R 16.90 min), 499 (t_R 17.29 min) and 553 (t_R 18.76 min) correspond, undoubtedly, to uliginosin A (**1**), isouliginosin B (**2**), hyperbrasilol B (**3**), uliginosin B (**4**) and hyperbrasilol B (**5**) as confirmed by NMR spectroscopy. Therefore, these samples were further submitted to UPLC-Q-TOF-MS analysis and direct infusion Q-TOF-MS to further determine the structure of the observed minor peaks at *m/z* of 515 (**1a**), 513 (**2a**), 567 (**3a**), 513 (**4a**) and 567 (**5a**). The **Table 2** shows the retention times, molecular and fragment ions from each MS/MS spectrum of compounds **1 - 5** and **1a - 5a** (spectra provided as Supplementary Information). MS/MS spectra, in positive mode, of dimeric acylphloroglucinols **1 - 5** indicate that the methylene bridge, that connects the filicinic acid and the phloroglucinol monomers, is the main fragmentation target (**Table 2**). The minor pair peaks **1a - 5a** were identified based on the MS/MS fragmentation pattern of the five NMR identified compounds **1** – **5** (**Table 3**, **Table 4**) and on the criteria that the MS/MS spectra of a dimeric acylphloroglucinol should reveal the *m/z* of the two monomers composing the diacylphloroglucinol. For example the MS/MS spectra in positive mode of **1** (*m/z* 501) (**Fig. 2**) displayed the ions at *m/z* of 277 and 265 corresponding to 3'-prenylphlorisobutyrophenone (*m/z* 265 = M - 236, neutral loss of 3',3'-dimethyl-6'-oxophlorisobutyrophenone with a methyl group fragment of the methylene bridge) and to 3'-prenylphlorisobutyrophenone (*m/z* 277 = M - 224, neutral loss of 3',3'-dimethyl-6'-oxophlorisobutyrophenone) with a methyl group fragment of the methylene bridge, respectively. Similarly, the homologue **1a** (*m/z* 515) displays the ions at *m/z* of 279 and 291 corresponding to the phloroglucinol moiety and a neutral loss of the filicinic acid moiety (M-

236 and M-224, respectively). Similar observations were found in the MS/MS fragmentation profiles of **2 - 5** and **2a - 5a**, indicating the location of an additional fragment, corresponding to 14 mass units, in the phloroglucinol moiety of compounds **1a - 5a** (**Table 3**).

The presence of higher homologues for **1** and **4** isolated from *H. uliginosum* was mentioned by Parker and Johnson 1968, who were unable to remove and identify this minor compound. Likewise, the isolation of compounds **1 - 5** from Peruvian *Hypericum* species were previously reported (Ccana-Ccapatinta et al. 2013), and here these compounds were shown to be in mixture with minor amounts of their correspondent M + 14 higher homologues. These minor compounds (**1a - 5a**) are here identified by chromatographic (HPLC, UPLC), spectroscopic (UV) and spectrometric (MS) evidence (**Fig. 1**, **Table 2**, **Table 4**). NMR analysis was not performed on these minor compounds because of the difficulty to separate these compounds in quantities sufficient for NMR experiments (Crispin et al. 2013). The difference in 14 mass units undoubtedly corresponds to an additional methylene group as part of the phloroglucinol moiety of compounds **1a - 5a** (**Fig. 1**, **Table 2**). The *iso*-butyryl side chain as part of the phloroglucinol moiety is found in hyperforin, while the 2-methylbutyryl side chain is observed in its M + 14 higher homologue adhyperforin (Maisenbacher and Kovar 1992). The levels of adhyperforin in *H. perforatum* is approximately 1/10 those of hyperforin (Jensen et al. 2001) that is in concordance with the minor levels found for compounds **1a - 5a** compared to their homologues **1 - 5**, even from different batches and from different plant sources (data not shown). A variety of M + 14 homologues with the *iso*-butyryl and 2-methylbutyryl acyl side chains are reported from *H. empetrifolium* (Schmidt et al. 2012a, 2012b), *H. chinense* (Abe et al. 2012) *H. cohaerens* (Liu et al. 2013) *H. beanii* (Shiu and Gibbons, 2006) among many other species. The structures proposed for **1a - 5a** as homologues of **1 - 5** (**Fig. 1**) are further supporter by the biosynthetically feasibility of their plausible precursors 2-methylbutyrylphloroglucinol and *iso*-butyrylphloroglucinol, respectively (Crockett et al. 2008). Valine and isoleucine are likely primary metabolite precursors involved in the biosynthesis of the *iso*-butyryl and 2-methylbutyryl acyl side chains of the phloroglucinol moiety as demonstrated in the biosynthesis of hyperforin and adhyperforin in shoot cultures of in *H. perforatum* (Karppinen et al. 2007). The structure of compounds **1a - 5a** and its natural occurrence in some *Hypericum* species (**Table 4**) are here presented for the first time.

Chemical fingerprinting of n-hexane extracts

The individual and overlaid HPLC-DAD chromatograms of the *n*-hexane extracts of six Peruvian *Hypericum* species, using the above mentioned HPLC fingerprint method, are shown in **Fig. 3**. Two popular clustering algorithms, hierarchical clustering analysis (HCA) and principal component analysis (PCA), were employed to investigate compositional similarities of the fingerprints.

In order to find some characteristic constituents which can serve as ideal taxonomic markers PCA was conducted. The score plot on the first two principal components (2PCs, 90.2% of variance explained) is presented in the **Fig. 3C**. Six peaks assigned as **a** - **f** were identified to explain 90.2 % of total variance by PCA. The peak **a** (**4**, uliginosin B) was certainly the main peak among the six samples analyzed. The chemotaxonomic importance of this compound in *Hypericum* species from section *Brathys* and *Trigynobrathys* was previously verified (Ferraz et al. 2002; Nör et al. 2004) and here further highlighted for six Peruvian *Hypericum* species. Peak **b - f** were located in the region of 5 – 15 min of the fingerprint chromatogram and the complete identity of these peaks are unknown. The content of these main peaks (**a** - **f**) as determined by PCA, dimeric acylphloroglucinol **1** - **5**, their M + 14 higher homologues **1a** - **5a,** and also some other minor common peaks (**u** - **z**) were quantified in equivalents of **5** and the result are shown in **Table 4**.

The HCA result is illustrated in the **Fig 3D**. *Hypericum andinum* was seen to be highly similar to *H. brevistylum* and those together with *H. decandrum*, *H. laricifolium* and *H. silenoides* were grouped in one cluster due to their similar fingerprint profiles, while *H. aciculate* was individually placed in another cluster. The present research included four species from section *Brathys* (*H. aciculare*, *H. andinum*, *H. decandrum* and *H. laricifolium*) and two from section *Trigynobrathys* (*H. brevistylum* and *H. silenoides*), which could not be differentiated at the sectional level by HCA. The number of species analyzed in this research was low compared to the total number of species described for these two sections (88 and 59). However, this study could serve as a framework for further studies exploring the chemotaxonomic utility of HPLC fingerprints of lipophilic extracts rich in acylphloroglucinol derivatives.

Dimeric acylphloroglucinol rich n-hexane extracts

With the aid of our reference substances, it was possible to identify peaks in the region of 3 - 4 min (**1** and **1a**) and 15 - 20 min (**2 - 5** and **2a -5a**) of the fingerprint chromatograms (**Fig. 3**). However, the region corresponding to 5 - 15 min and, in a lesser extent, 20 - 25 min of the chromatograms exhibited many other unidentified peaks. Lipophilic extracts of *Hypericum* species from section *Brathys* and *Trigynobrathys* are hypothesized to be mainly constituted by dimeric acylphloroglucinols (Barros et al. 2013; Ccana-Ccapatinta et al. 2013). As a first approach to determine the nature of these unidentified peaks, a comparison of their UV spectra with those UV spectra of identified peaks were conducted in each sample and subsequently analyzed by PCA and HCA (Supplementary information). These UV spectra overlaid of identified and unidentified peaks showed that most of these peaks display characteristically three-peaked UV spectra with absorption maxima in turn of 220, 300 and 350 nm as determined by PCA. This initial observation, in addition to the high percentage of similarity (more than 65%, determined by HCA), suggested that these peaks would correspond to dimeric acylphloroglucinols. Previously, three peaked UV spectra were suggested as indicative of dimeric acylphloroglucinols in *H. gentianoides* (Hillwig et al. 2008).

Further efforts were carried out to obtain structural information of the unidentified mayor (**b - f**) and some minor common (**u - z**) peaks. LC-ESI-MS analysis revealed *m/z* values above 500 (**Table 4**), compatible with the proposition that these peaks must correspond to dimeric acylphloroglucinols because they exceeded the minimum weight for a diacylphloroglucinol (404 D for albaspidin AA) (Crispin et al. 2013, Ccana-Ccapatinta et al. 2013). The MS/MS spectra of peaks **b - f** and **u - z** acquired by UPLC-Q-TOF-MS displayed a characteristically fragmentation pattern as observed for compounds **1 - 5** and **1a - 5a** (Supplementary information). As a general trend, the beak down into two roughly equal parts and the characteristic neutral loss of a 3',3'-dimethyl-6'-oxophlorisobutyrophenone (M-224 and M-236) was clearly seen in the MS/MS spectra of peaks **b - f** and **u – z**. These observations further suggested the possible dimeric nature of these potential acylphloroglucinol derivatives.

The occurrence of regioisomers in the *n*-hexane extracts of Peruvian *Hypericum* species is illustrated in **Fig. 4** and **Fig. 5**. The monitoring at *m/z* of 499, 513, 553 and 567 showed the presence of the regioisomeric compounds corresponding to **2** and **4** (**Fig. 5B**), **3** and **5** (**Fig. 5C**), **2a** and **4a** (**Fig. 5D**), **3a** and **5a** (**Fig. 5E**). The certainly assignment of these

regioisomers in the BIP chromatograms were achieved by cochromatography with the reference substances, comparison of MS/MS spectra and analysis of the chromatographic elution pattern. In fact, the distinction of these regioisomers solely by MS/MS data was not possible because of the highly similarity of their fragmentation patterns. Compounds **2** and **4**, which differ in the cyclisation pattern of the pyran ring, are distinguished each other by comparison of their hydroxyl resonances in the ^{1}H and HMBC NMR spectra. The **Fig. 4** displays the monitoring at *m/z* of 517, 531, 585 and 599 that showed peaks corresponding to **v** and **c** (**Fig. 5B**), **w** and **x** (**Fig. 5C**), **d** and **e** (**Fig. 5D**), then **y** and **z** (**Fig. 5E**). The possible identities of those dimeric acylphloroglucinols are presented in **Fig. 6** based on their molecular ions, fragmentation pattern, and biosynthetically possibilities.

Antichemotactic activity of n-hexane extracts and its main component

The effect of *n*-hexane extracts of six Peruvian *Hypericum* species on the *in vitro* chemotaxis of polymorphonuclear neutrophils is illustrated in **Table 5**. The results showed that the extracts displayed significant inhibition of the LPS-induced chemotaxis on rat PMNs. The effect was dose-dependent in the range of concentration assayed. Inhibition values above 60% on the same antichemotactic assay were observed in the dose range of 10 - 0.3 µg/mL of *n*-hexane extracts from *H. carinatum, H. linoides* and *H. myrianthum*, southern Brazilian *Hypericum* species (Barros et al. 2013). In the present study inhibition values of 100% were observed at doses of 10 and 1 µg/ml of *H. aciculate*, *H. laricifolium* and *H. silenoides*. These species together with *H. decandrum* displayed the highest approximate total content of dimeric acylphloroglucinols. This observation together with the greater chemical diversity as seen in their fingerprint profiles can explain the greater values of PMN chemotaxis imbibition observed for these four extracts compared to *H. andinum* and *H. brevistylum*. Uliginosin B (**4**) was the common compound among the six samples assayed, and as a general trend its concentrations were the highest of dimeric acylphloroglucinols (**Table 4**). This compound (as an 3:1 mixture with its M + 15 higher homologue) was shown to inhibit considerably the *in vitro* PMN chemotaxis with a IC_{50} of 0.26 µM (**Table 6**). Fractions and uliginosin A from *H. gentianoides* were shown to potently inhibit the PG(E_2) production at 2.0 and 2.6 µM (Huang et al. 2011). The concentrations of uliginosin A in the extracts were shown to be low that could not count for the overall effect observed. Event thought, the structural similarity of uliginosin A with uliginosin B and the rest of dimeric acylphloroglucinols suggest the

inhibition of PG(E_2) production as one possible mechanism of the observed inhibitory effect of the *n*-hexane extracts and its main component uliginosin B (**4**) on the PMN chemotaxis. Further studies are needed to elucidate the individual contribution of each dimeric acylphloroglucinol in the overall effect observed for the *n*-hexane extracts.

Conclusions

The *n*-hexane extracts of Peruvian *Hypericum* species, collected in high altitude Páramo habitats, are complex mixtures of dimeric acylphloroglucinols derivatives. The presence of M + 14 higher homologues and regioisomers could be discriminated by means of HPLC-DAD, LC-ESI-MS and UPLC-Q-TOF-MS. The naturally occurrence of five previously undescribed acylphloroglucinols (M + 14 higher homologues) is presented and the presence of other compounds (mixture of regioisomers), identified by the analysis of their fragmentation pattern, is presented. These extracts and its main dimeric acylphloroglucinol component were able to potently inhibit the LPS-induced chemotaxis on rat PMNs. These results suggest that the *n*-hexane extracts of *Hypericum* species from sections *Brathys* and *Trigynobrathys* are potential sources of new anti-inflammatory molecules.

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Table 1 Collection localities of Peruvian *Hypericum* species

Species	Collection locality	Elevation[a]	Collection Date	Voucher number
H. aciculare	Huancabamba, Piura, Peru	3100	February 2012	Ccana-Ccapatinta *et al*., 10
H. andinum	Amparaes, Cuzco, Peru	3432	May 2008	Ccana-Ccapatinta 06
H. brevistylum	Paqchaq, Cuzco, Peru	3573	March 2008	Ccana-Ccapatinta 04
H. decandrum	Luya, Amazonas, Peru	3120	February 2012	Ccana-Ccapatinta *et al*., 12
H. laricifolium	Cumbemayo, Cajamarca, Peru	3450	November 2009	Ccana-Ccapatinta 08
H. silenoides	Pumahuanca, Cuzco, Peru	3189	March 2008	Ccana-Ccapatinta 02

[a]meters above sea level

Table 2 Retention times values, molecular and MS/MS ions of dimeric acylphloroglucinols (**1-5**) and its M+14 higher homologues (**1a-5a**) as identified by HPLC and UPLC-Q-TOF-MS

Standard	HPLC t_R (min)	UPLC t_R (min)	Abbreviation/ Common name	*m/z*	Fragment ions
1	3.25	1.89	uliginosin A	501	191, 209, 225, 265, 277, 413, 445, 483
1a	3.75	2.17	[3'3'me6'oxoPIB]-[3'pren4mePIB]	515	191, 209, 237, 279, 291, 425, 459, 497
2	15.26	3.15	isouliginosin B	499	193, 219, 245, 263, 275
2a	16.27	3.32	[3'3'me6'oxoPIB]-[6meb22meC57diol]	513	205, 245, 263, 275, 277, 289
3	16.90	3.49	isohyperbrasilol B	553	69, 223, 257, 263, 275, 276
3a	17.95	3.63	[3'pren3'me6'oxoPIB]-[6meb22meC57diol]	567	69, 205, 223, 257, 263, 271, 289
4	17.29	3.51	uliginosin B	499	219, 237, 245, 263, 275
4a	18.39	3.63	[3'3'me6'oxoPIB]-[8meb22meC57diol]	513	233, 245, 263, 275, 277, 289
5	18.76	3.73	hyperbrasilol B	553	69, 205, 223, 245, 257, 263, 275
5a	19.90	3.88	[3'pren3'me6'oxoPIB]-[8meb22meC57diol]	567	69, 223, 259, 271, 275, 277, 289

Table 3 Empirical formulas, molecular ions, and designation of dimeric acylphloroglucinols (**1-5**) and its corresponding M+14 higher homologues (**1a-5a**), as identified by high-resolution Q-TOF mass analysis

Standard	Empirical formula	Predicted m/z	Measured m/z	Mass difference (ppm)	Systematic name
1	C28H37O8	501.2488*	501.2463	2.5	3,5-dihydroxy-2-isobutyryl-4,4-dimethyl-6-(2,4,6-trihydroxy-3-isobutyryl-5-(3-methylbut-2-enyl)benzyl)cyclohexa-2,5-dienone
1a	C29H39O8	515.2645	515.2677	-3.2	3,5-dihydroxy-2-isobutyryl-4,4-dimethyl-6-(2,4,6-trihydroxy-3-(2-methylbutanoyl)-5-(3-methylbut-2-enyl)benzyl)cyclohexa-2,5-dienone
2	C28H35O8	499.2332	499.2306	2.6	2-((5,7-dihydroxy-6-isobutyryl-2,2-dimethyl-2Hchromen-8-yl)methyl)-3,5-dihydroxy-6-isobutyryl-4,4-dimethylcyclohexa-2,5-dienone
2a	C29H37O8	513.2488	513.2491	-0.3	2-((5,7-dihydroxy-2,2-dimethyl-6-(2-methylbutanoyl)-2Hchromen-8-yl)methyl)-3,5-dihydroxy-6-isobutyryl-4,4-dimethylcyclohexa-2,5-dienone
3	C32H41O8	553.2801	553.2800	0.1	2-((5,7-dihydroxy-6-isobutyryl-2,2-dimethyl-2Hchromen-8-yl)methyl)-3,5-dihydroxy-6-isobutyryl-4-methyl-4-(3-methylbut-2-enyl)cyclohexa-2,5-dienone
3a	C33H43O8	567.2958	567.2963	-0.5	2-((5,7-dihydroxy-2,2-dimethyl-6-(2-methylbutanoyl)-2Hchromen-8-yl)methyl)-3,5-dihydroxy-6-isobutyryl-4-methyl-4-(3-methylbut-2-enyl)cyclohexa-2,5-dienone
4	C28H35O8	499.2332	499.2320	1.2	2-((5,7-dihydroxy-8-isobutyryl-2,2-dimethyl-2Hchromen-6-yl)methyl)-3,5-dihydroxy-6-isobutyryl-4,4-dimethylcyclohexa-2,5-dienone
4a	C29H37O8	513.2488	513.2465	2.3	2-((5,7-dihydroxy-2,2-dimethyl-8-(2-methylbutanoyl)-2Hchromen-6-yl)methyl)-3,5-dihydroxy-6-isobutyryl-4,4-dimethylcyclohexa-2,5-dienone
5	C32H41O8	553.2801	553.2804	-0.3	2-((5,7-dihydroxy-8-isobutyryl-2,2-dimethyl-2Hchromen-6-yl)methyl)-3,5-dihydroxy-6-isobutyryl-4-methyl-4-(3-methylbut-2-enyl)cyclohexa-2,5-dienone
5a	C33H43O8	567.2958	567.2922	3.7	2-((5,7-dihydroxy-2,2-dimethyl-8-(2-methylbutanoyl)-2Hchromen-6-yl)methyl)-3,5-dihydroxy-6-isobutyryl-4-methyl-4-(3-methylbut-2-enyl)cyclohexa-2,5-dienone

* in positive mode

Table 4 Retention time values, molecular ions and content (%) of dimeric acylphloroglucinols (**1-5**), their correspondent M+14 higher homologues (**1a-5a**), main peaks as determined by PCA (**a-f**) and some minor common peaks (**u-z**) in the *n*-hexane extracts of Peruvian *Hypericum* species

	HPLC t_R (min)	**m/z (ESI-)**	**m/z (ESI+)**	**HACI**	**HAND**	**HBRE**	**HDEC**	**HLAR**	**HSIL**
1	3.25	499	501	0.5[a]	0.5	0.4	1.1	0.9	0.8
1a	3.75	513	515	0.4	0.4	0.5	0.8	0.4	0.6
b	6.26	567	569	1.3	0.4	Nd	1.9	9.6	Nd
u	7.04	567	569	+	+	+	2.5	1.4	+
v	8.32	515	517	1.2	1.1	1.5	1.3	2.4	5.2
f	9.28	583	585	+	+	Nd	0.7	3.5	Nd
w	9.55	529	531	1.2	0.4	0.9	1.1	+	1.3
c	10.56	515	517	2.5	1.0	2.0	1.8	3.2	6.7
d	11.33	583	585	2.1	1.1	1.1	1.8	1.8	1.1
x	11.56	529	531	+	+	1.3	+	+	+
y	12.43	597	599	2.5	0.9	0.7	nd	+	+
e	14.39	583	585	4.5	3.6	0.3	+	+	+
2	15.26	497	499	0.2	0.6	0.4	1.6	2.2	0.4
z	15.69	597	599	2.6	0.5	0.4	0.5	0.4	0.3
2a	16.27	511	-	0.6	+	+	1.3	+	+
3	16.90	551	-	nd	Nd	nd	nd	2.8	Nd
a(4)	17.29	497	499	6.8	5.9	8.5	8.6	9.1	9.1
3a	17.95	565	-	nd	Nd	nd	nd	0.6	Nd
4a	18.39	511	-	3.3	1.0	2.2	2.2	1.1	2.3
5	18.76	551	-	nd	+	nd	0.4	1.7	Nd
5a	19.89	565	-	nd	Nd	nd	nd	0.5	Nd
Approx. Total Content (Σ)				29.7	17.4	20.2	27.6	41.6	27.8

[a]Values are expressed as mean ± standard deviation of three assays; nd, not detected; +, presence, below limit of detection and/or overlapping signal peaks. Peruvian *Hypericum* species: *Hypericum aciculare* (HACI), *H. andinum* (HAND), *H. decandrum* (HDEC), *H. laricifolium* (HLAR), *H. brevistylum* (HBRE) and *H. silenoides* (HSIL)

Table 5 Effect of *n*-hexane extracts of some Peruvian *Hypericum* species, on the *in vitro* LPS-induced chemotaxis of rat polymorphonuclear neutrophils[a]

Sample	Concentration (µg/mL)	Migration (µm)	Migration inhibition (%)
HACI	10	0*	100
	1	0*	100
	0.1	38.4 ± 4.4*	50.8
	0.01	26.8 ± 1.0*	65.7
HAND	10	12.8 ± 0.4*	83.7
	1	11.0 ± 0.5*	85.9
	0.1	46.9 ± 3.9*	39.9
	0.01	50 .0 ± 5.6*	35.9
HBRE	10	2.6 ± 0.3*	96.7
	1	43.3 ± 1.6*	44.6
	0.1	63.8 ± 1.9*	18.3
	0.01	74.0 ± 1.4*	5.2
HDEC	10	8.2 ± 0.6*	89.5
	1	32.4 ± 3.5*	58.4
	0.1	26.5 ± 1.3*	66.1
	0.01	24.8 ± 1.4*	68.2
HLAR	10	0*	100
	1	0*	100
	0.1	30.3 ± 3.5*	61.1
	0.01	21.4 ± 2.2*	72.6
HSIL	10	0*	100
	1	0*	100
	0.1	27.3 ± 3.7*	65.0
	0.01	48.8 ± 2.3*	37.5

[a]Chemotaxis is presented as means ± SEM of leukocyte migration (µm). Positive control: lipopolysaccharide (LPS 13 µg/mL) from *E. coli* (LPS = 78.1 ± 2.6). *$p \leq 0.001$ compared to positive control (reference chemoattractant) LPS (ANOVA-Tukey's test). Peruvian *Hypericum* species: *Hypericum aciculare* (HACI), *H. andinum* (HAND), *H. decandrum* (HDEC), *H. laricifolium* (HLAR), *H. brevistylum* (HBRE) and *H. silenoides* (HSIL).

Table 6 Effect of uliginosin B on the *in vitro* chemotaxis of polymorphonuclear neutrophils[a]

Sample	Concentration (µM)	Migration (µm)	Migration inhibition (%)	IC_{50}
Uliginosin B	100	10.2 ± 0.4*	87.4	0.26 ± 0.07
	10	11.7 ± 0.9*	85.6	
	1	20.0 ± 0.1*	75.3	
	0.1	48.0 ± 3.6*	39.9	
	0.01	77.7 ± 3.1	4.3	
Indomethacin	28	38.0 ± 3.4*	44.9	

[a]Chemotaxis is presented as means ± SEM of leukocyte migration (µm). Positive control: lipopolysaccharide (LPS 13 µg/mL) from *E. coli* (LPS = 81.2 ± 3.2). *$p \leq 0.001$ compared to positive control (reference chemoattractant) LPS (ANOVA-Tukey's test)

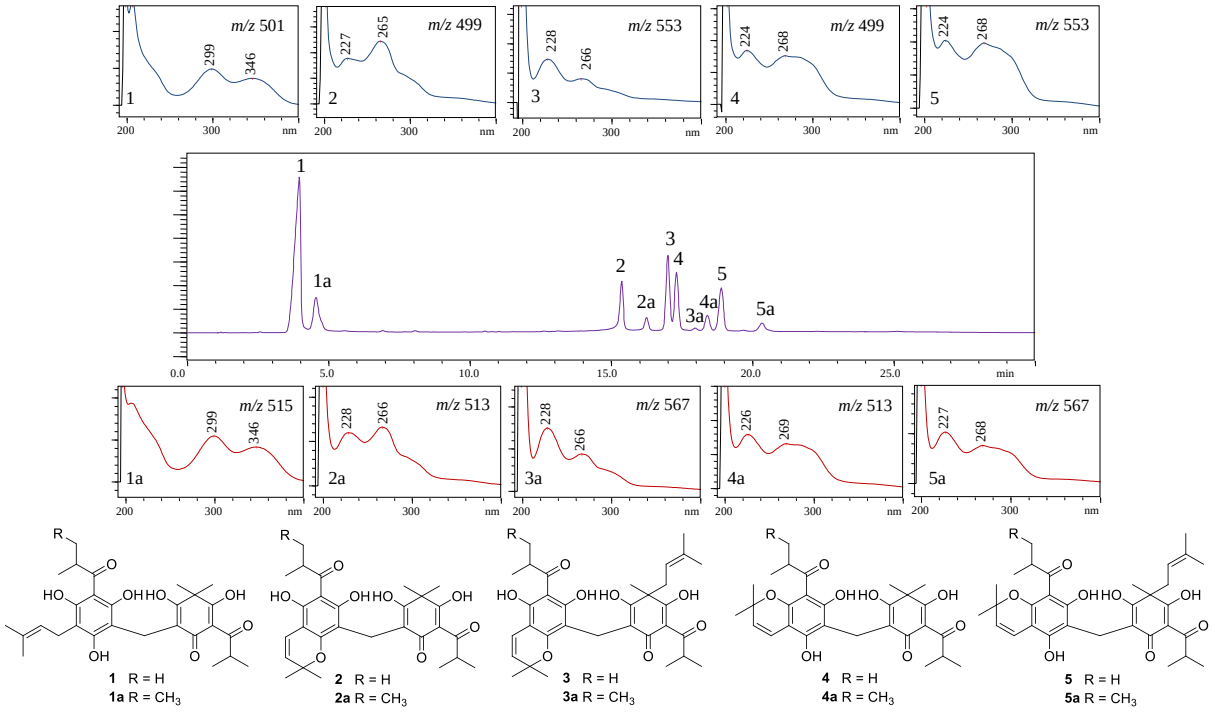

Fig. 1 HPLC-DAD chromatogram, UV spectra, MS values, and chemical structures of dimeric acylphloroglucinols (**1-5**) and their correspondent M+14 higher homologues (**1a-5a**) as determined by HPLC-DAD associated with LC-ESI-MS and UPLC-Q-TOF-MS

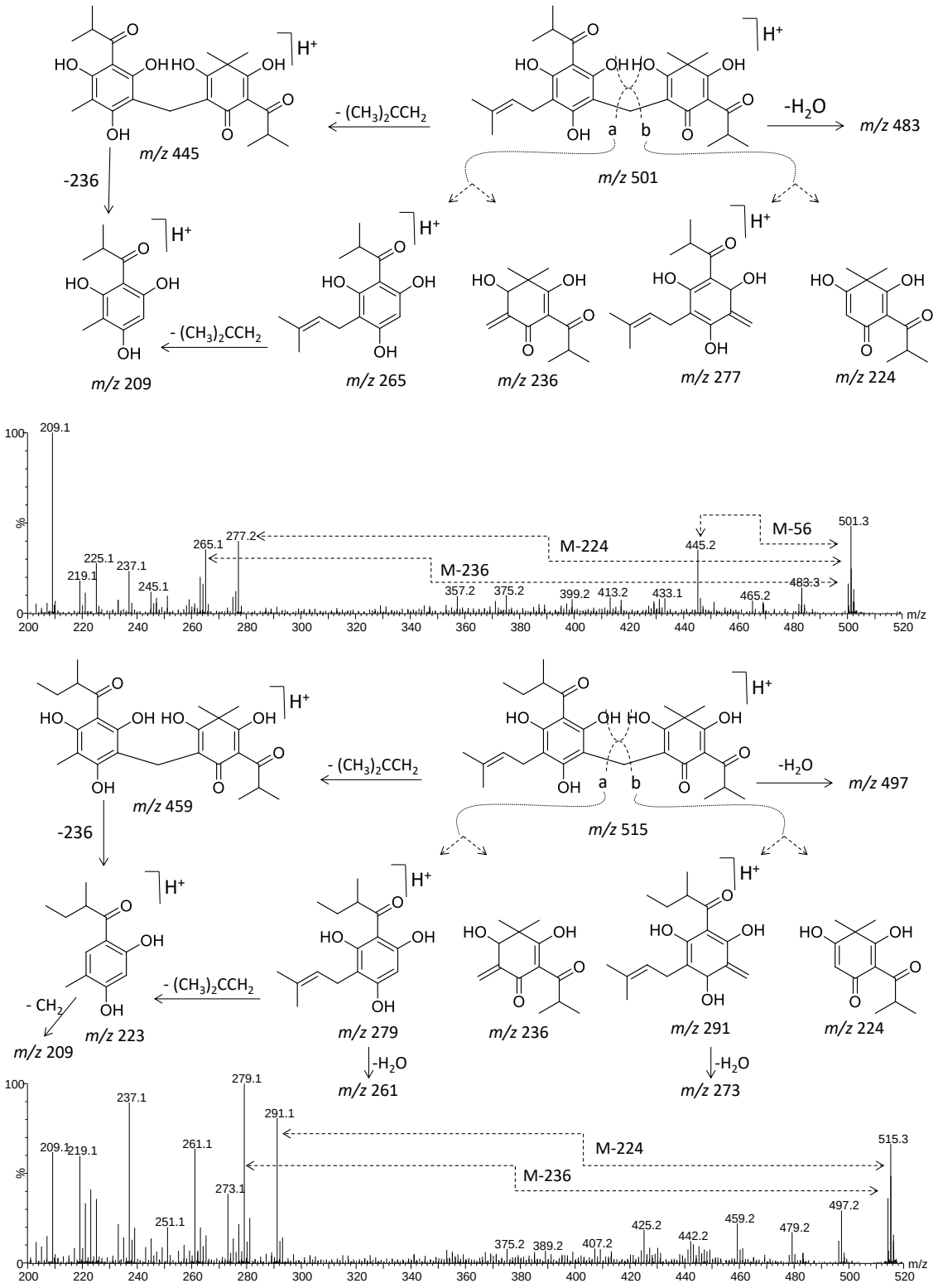

Fig. 2 Fragmentation pattern of **1** and **1a** in positive mode and expansion of their MS/MS spectra.

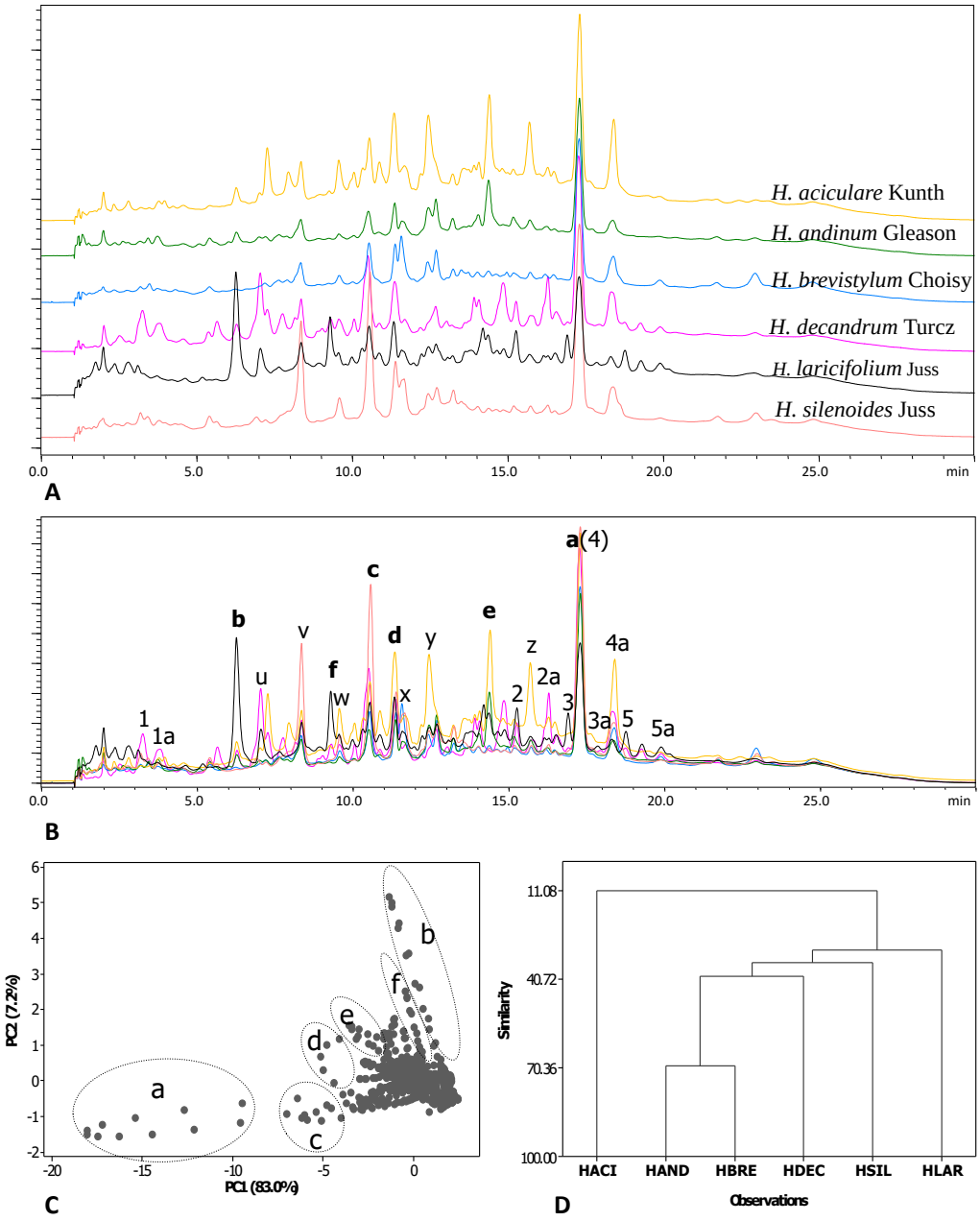

Fig. 3 HPLC-DAD chromatograms of Peruvian *Hypericum* spp. (A) individual and (B) overlaid chromatograms of the *n*-hexane extracts from *Hypericum* spp., monitored at 350 nm. (C) Score plot (PC1xPC2) obtained from PCA analysis of the *n*-hexane extract chromatograms. (D) Dendrogram obtained from HCA analysis of the *n*-hexane extract chromatograms. *Note:* peaks assigned as **1** to **5** and **1a** to **5a** correspond to known dimeric acylphloroglucinols and its M+15 higher homologues, respectively; **a** to **f** represent main peaks in the HPLC-PDA chromatograms of Peruvian *Hypericum* spp., responsible for chemical differentiation of *n*-hexane extracts as determined by PCA; **u** to **z** are other common minor peaks.

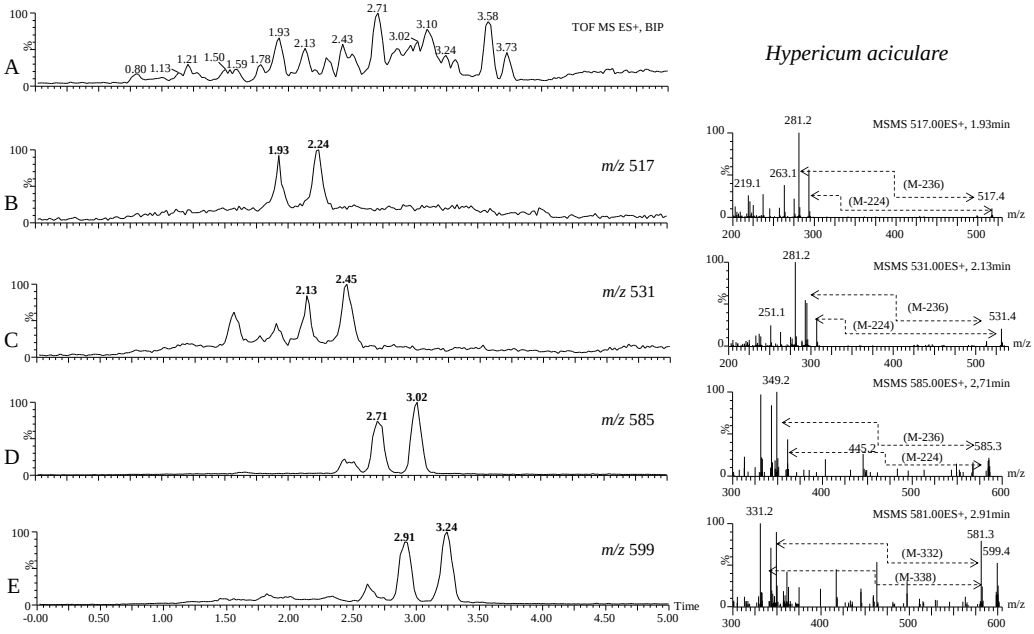

Fig. 4 UPLC-Q-TOF-MS chromatogram of the *n*-hexane extract of *H. aciculare*. (A) Base ion peak (BIP) chromatogram in positive mode (*m*/*z* range 50-1000). (B-E) reconstructed BIP chromatograms at *m*/*z* values of, 517 (**v**, **c**), 531 (**w**, **x**), 585 (**d**, **e**) and 599 (**y**, **z**) and their correspondent MS/MS spectra with key fragment values.

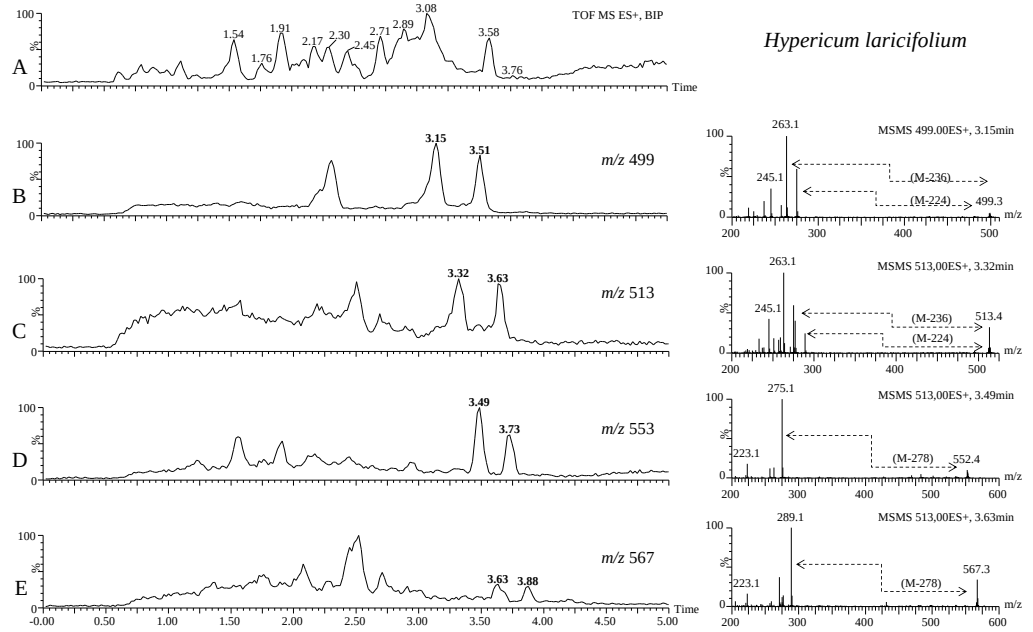

Fig. 5 UPLC-Q-TOF-MS chromatogram of the *n*-hexane extract of *H. laricifolium*. (A) Base peak ion (BIP) chromatogram in positive mode (*m*/*z* range 50-1000). (B-E) reconstructed BIP chromatograms at *m*/*z* values of 499 (**2**, **4**), 513 (**2a**, **4a**), 553 (**3**, **5**) and 567 (**3a**, **5a**) and their correspondent MS/MS spectra with key fragment values.

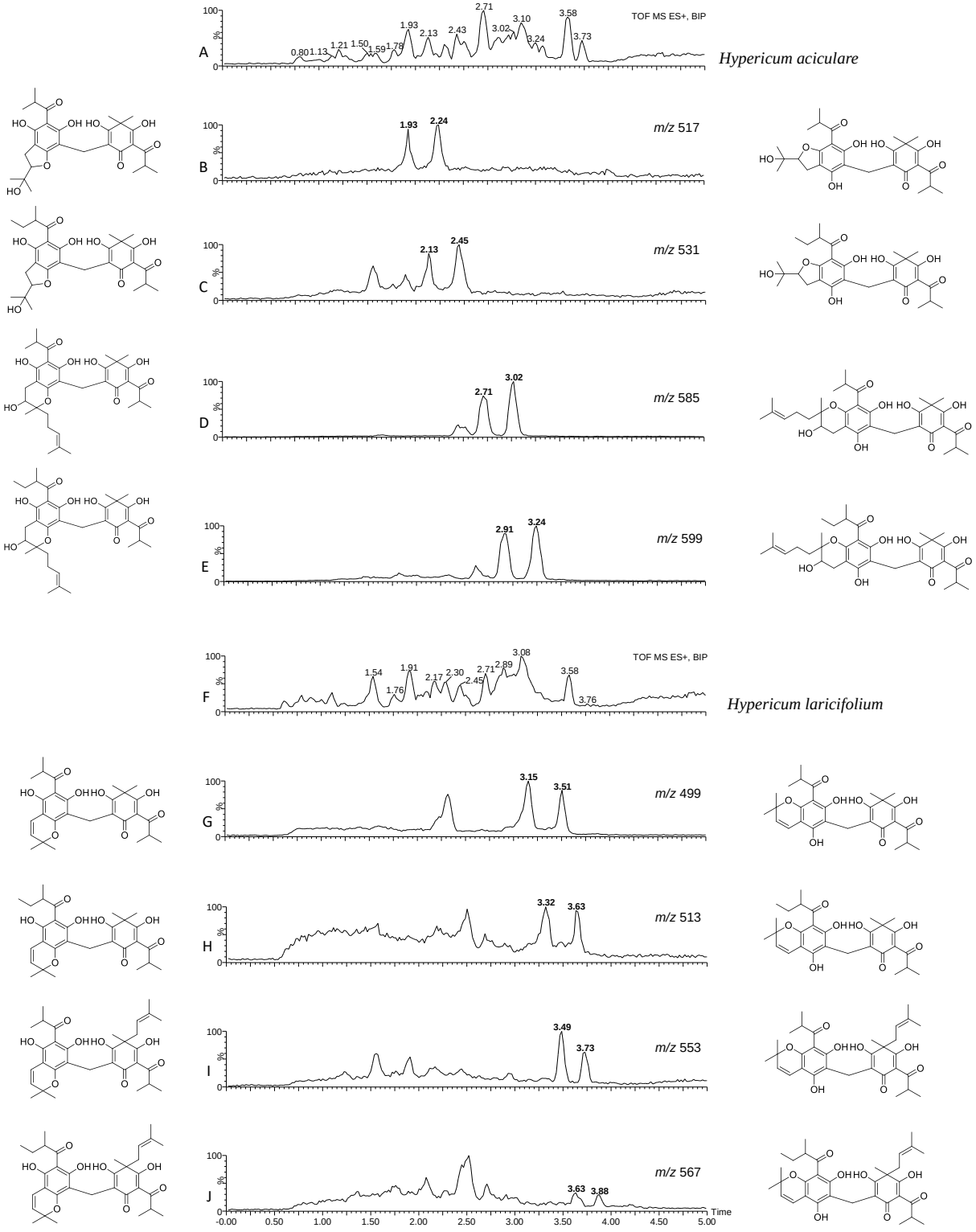

Fig. 6 Possible identify of dimeric acylphloroglucinols based on their molecular and fragmentation pattern, fragment ions and biosynthetically possibilities.

Dimeric acylphloroglucinol rich *n*-hexane extracts of Peruvian *Hypericum* species with antichemotactic activity: fingerprint and chemometrics

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Supplementary information

Table. S1. Precision, repeatability, intra- and inter-day reproducibility of the HPLC-DAD fingerprint method based on the relative standard deviation (RSD) of retention times (t_R) and peak concentration (PC) of isohyperbrasilol B, uliginosin B and hyperbrasilol B determined in a samples of *Hypericum laricifolium.*

Parameter	**Sample Concentration**	**Isohyperbrasilol B**		**Uliginosin B**		**Hyperbrasilol B**	
		t_R	Peak Area	t_R	Peak Area	t_R	Peak Area
Precision RSD (%)	HLAR 1000 µg/ml	1.9	3.6	1.9	2.2	1.9	5.6
Repeatability RSD (%)	HLAR 1000 µg/ml	2.0	7.4	2.0	2.1	1.9	6.2
Intra-day reproducibility RSD (%)	HLAR 100 µg/ml	1.1	3.4	1.1	3.6	1.2	4.2
	HLAR 500 µg/ml	0.7	1.3	0.7	2.4	0.7	3.3
	HLAR 1000 µg/ml	0.3	1.9	0.4	0.3	0.4	0.2
	HLAR 2000 µg/ml	0.3	1.2	0.3	0.4	0.3	1.1
Inter-day reproducibility RSD (%)	HLAR 500 µg/ml	3.4	8.7	3.6	6.1	3.5	8.7
	HLAR 1000 µg/ml	1.9	3.2	1.9	2.6	1.9	4.4

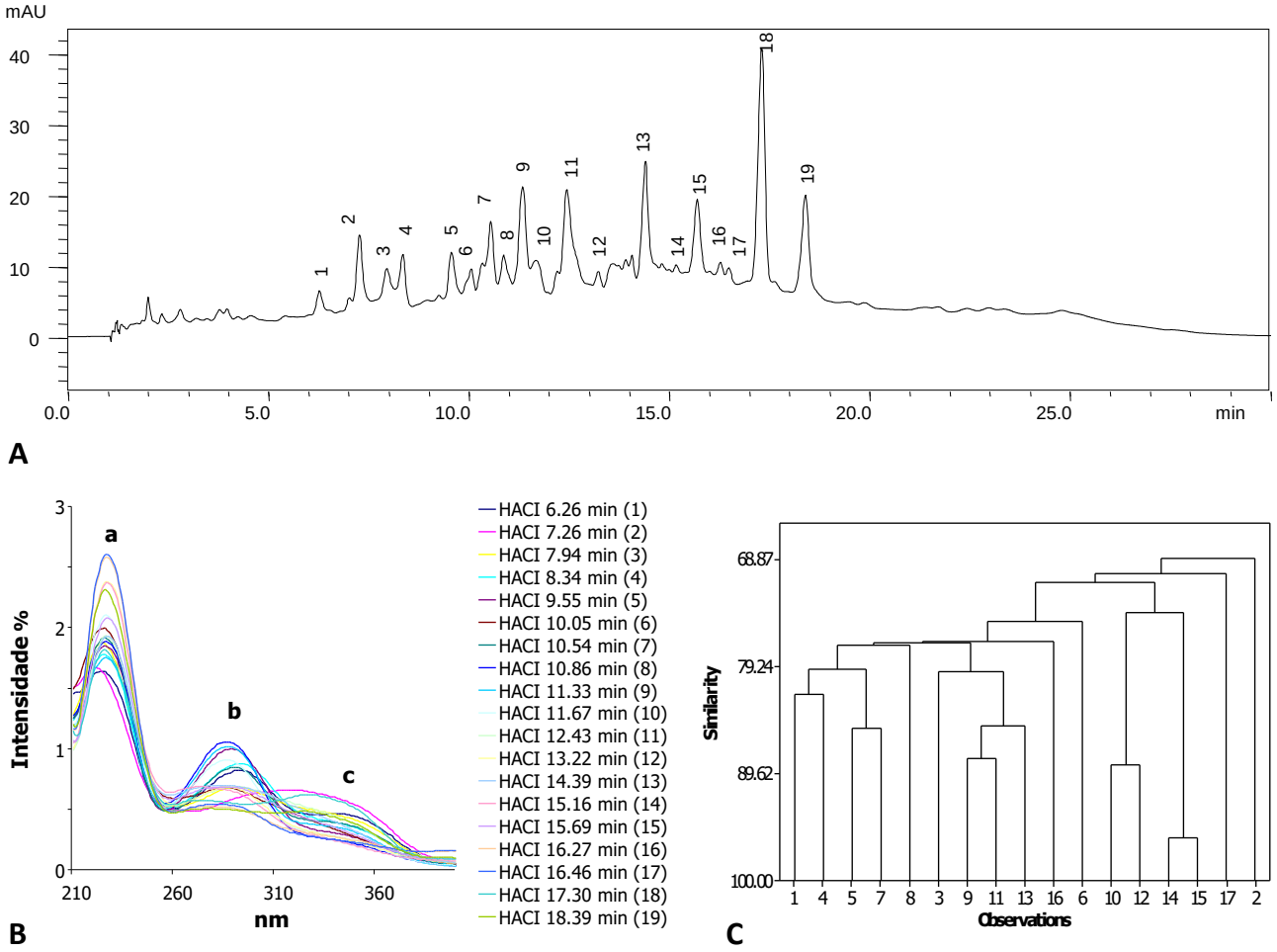


Fig. S1 (A) HPLC-DAD chromatogram of the *n*-hexane extract of *Hypericum aciculare* (HACI), at 350 nm. (B), UV-Vis spectra overlaid of major peaks presents in the HPLC-DAD chromatogram. (C) Dendrogram obtained from HCA analysis of UV-Vis spectra of major peaks presents in the HPLC-DAD chromatogram. *Note:* a – c represent the main peaks of the UV-Vis spectra.

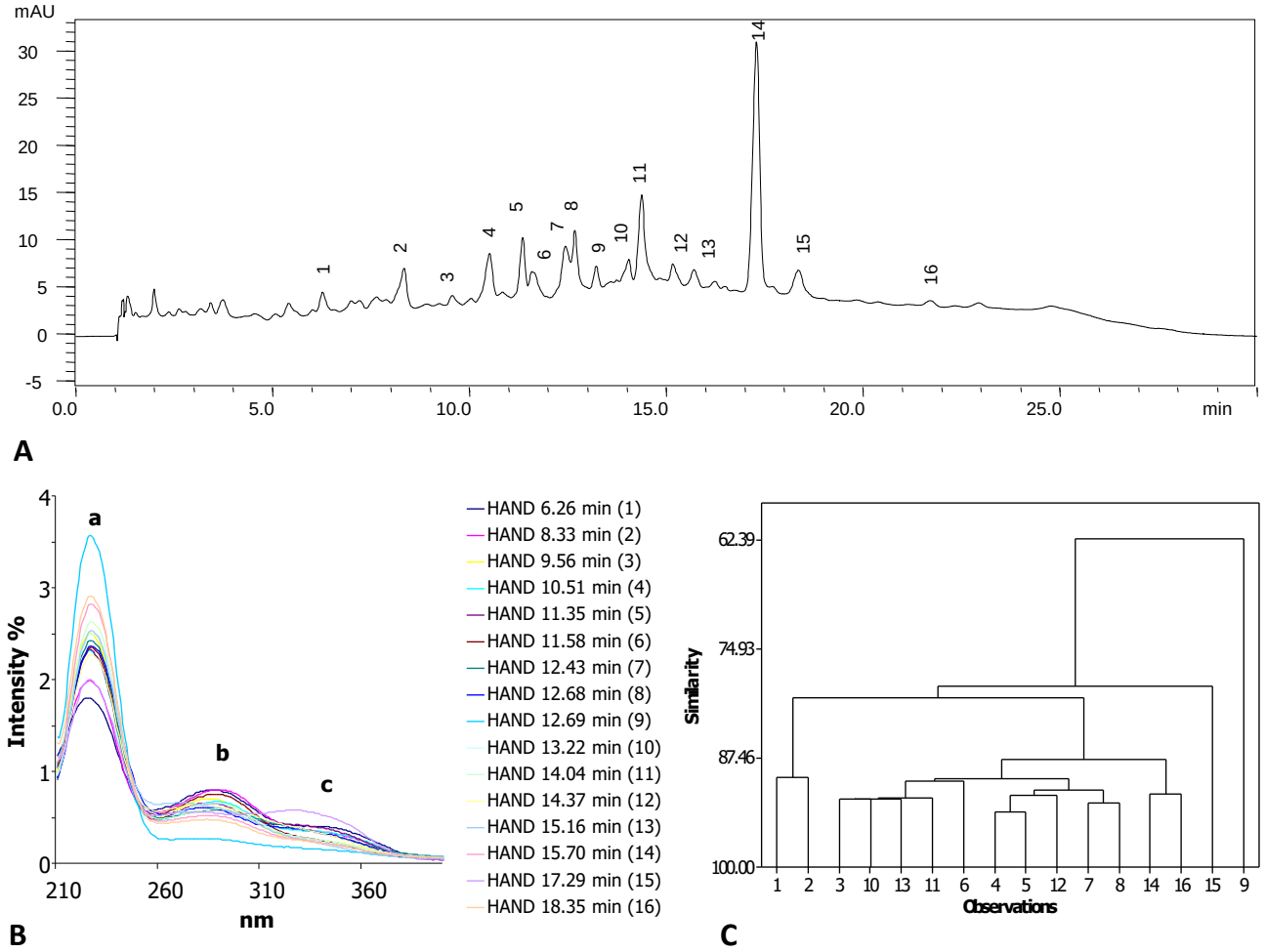


Fig. S2 (A) HPLC-DAD chromatogram of the *n*-hexane extract of *Hypericum andinum* (HAND), at 350 nm. (B) UV-Vis spectra overlaid of major peaks presents in the HPLC-DAD chromatogram. (C) Dendrogram obtained from HCA analysis of UV-Vis spectra of major peaks presents in the HPLC-DAD chromatogram. *Note:* a – c represent the main peaks of the UV-Vis spectra.

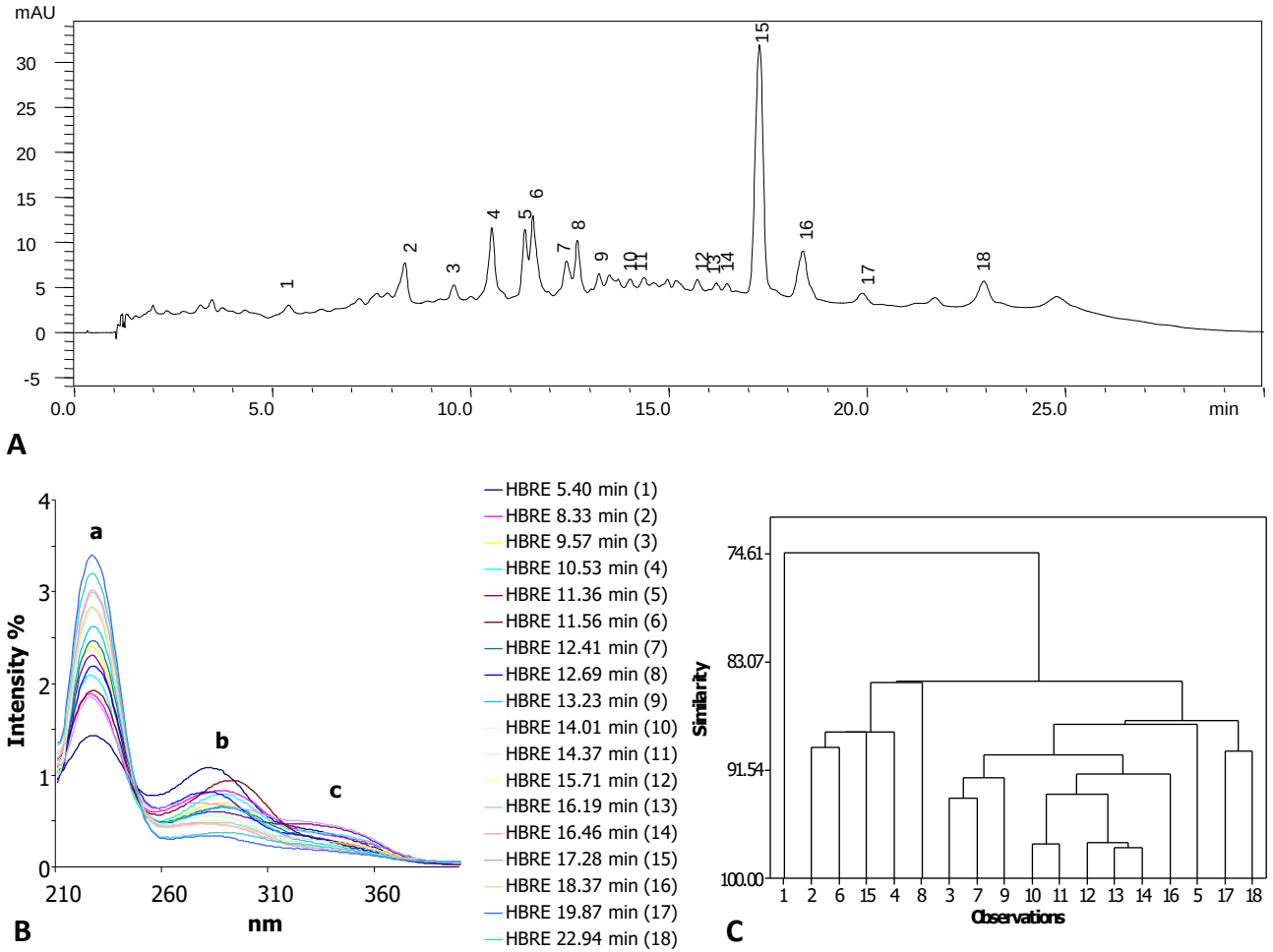


Fig. S3 (A) HPLC-DAD chromatogram of the *n*-hexane extract of *Hypericum brevistylum* (HBRE), at 350 nm. (B) UV-Vis spectra overlaid of major peaks presents in the HPLC-DAD chromatogram. (C) Dendrogram obtained from HCA analysis of UV-Vis spectra of major peaks presents in the HPLC-DAD chromatogram. *Note:* a – c represent the main peaks of the UV-Vis spectra.

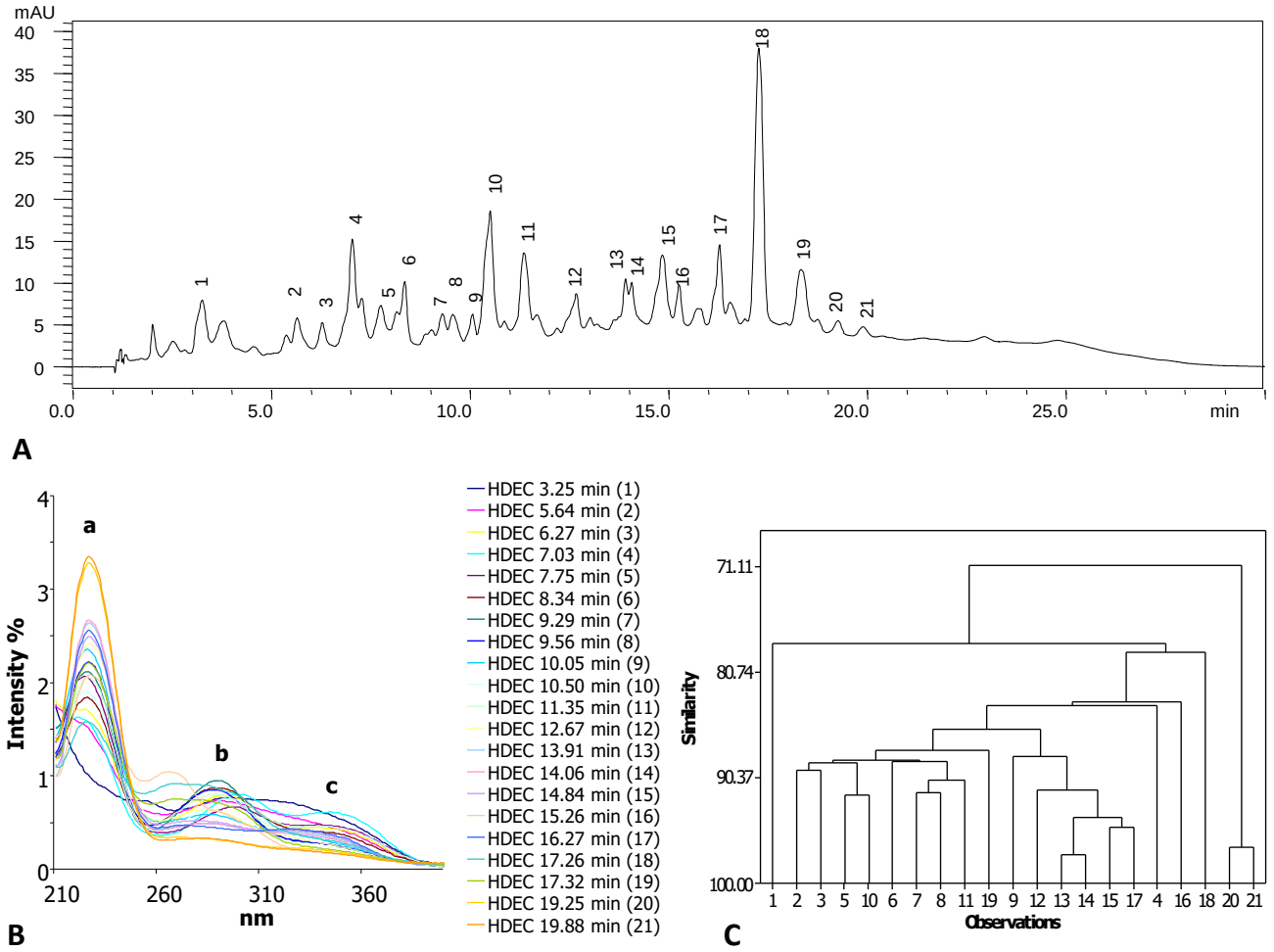


Fig. S4 (A) HPLC-DAD chromatogram of the *n*-hexane extract of *Hypericum decandrum* (HDEC), at 350 nm. (B) UV-Vis spectra overlaid of major peaks presents in the HPLC-DAD chromatogram. (C) Dendrogram obtained from HCA analysis of UV-Vis spectra of major peaks presents in the HPLC-DAD chromatogram. *Note:* a – c represent the main peaks of the UV-Vis spectra.

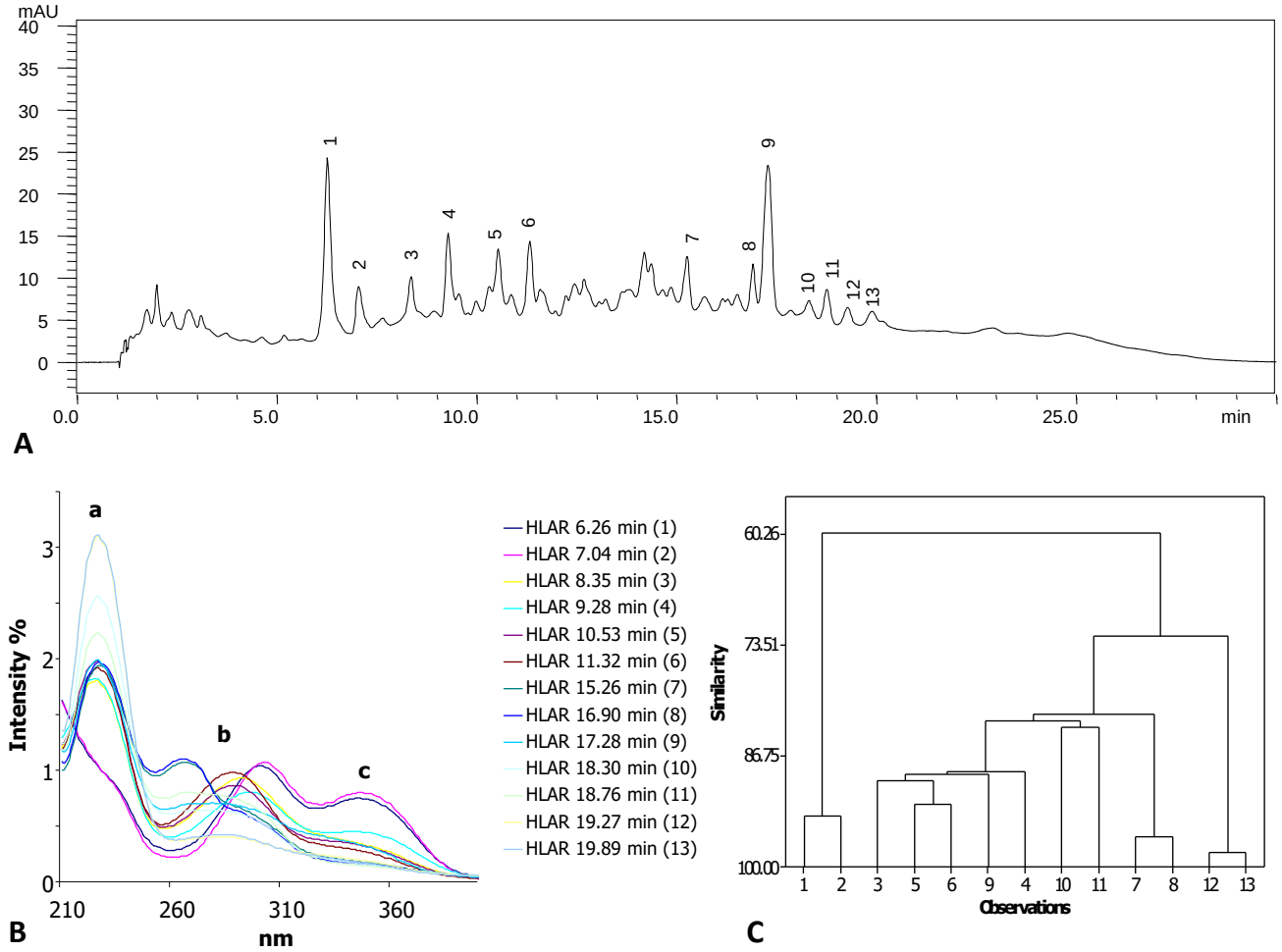


Fig. S5 (A) HPLC-DAD chromatogram of the *n*-hexane extract of *Hypericum laricifolium* (HDEC), at 350 nm. (B) UV-Vis spectra overlaid of major peaks presents in the HPLC-DAD chromatogram. (C) Dendrogram obtained from HCA analysis of UV-Vis spectra of major peaks presents in the HPLC-DAD chromatogram. *Note:* a – c represent the main peaks of the UV-Vis spectra.

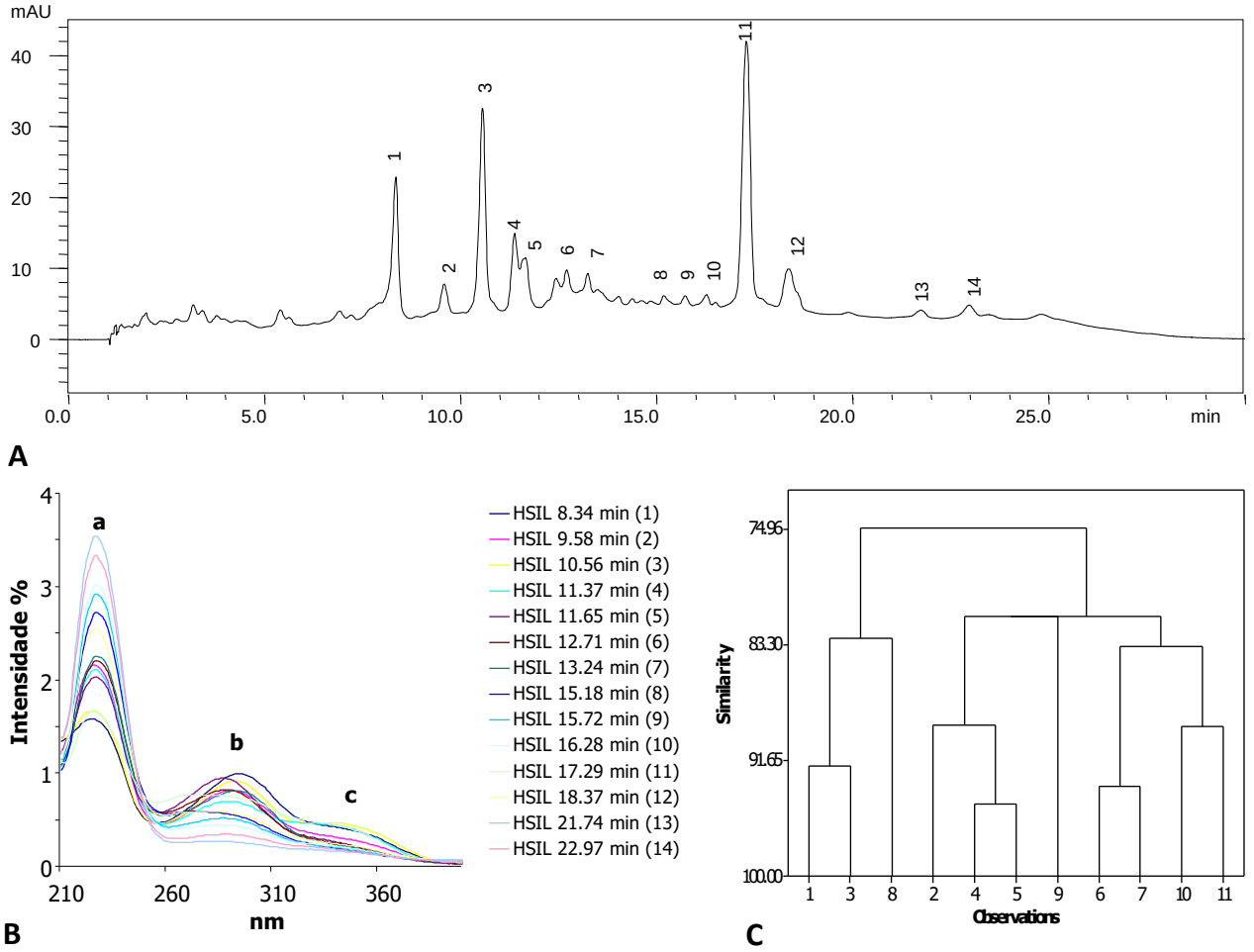


Fig. S6 (A) HPLC-DAD chromatogram of the *n*-hexane extract of *Hypericum silenoides* (HSIL), at 350 nm. (B) UV-Vis spectra overlaid of major peaks presents in the HPLC-DAD chromatogram. (C) Dendrogram obtained from HCA analysis of UV-Vis spectra of major peaks presents in the HPLC-DAD chromatogram. *Note:* a – c represent the main peaks of the UV-Vis spectra.

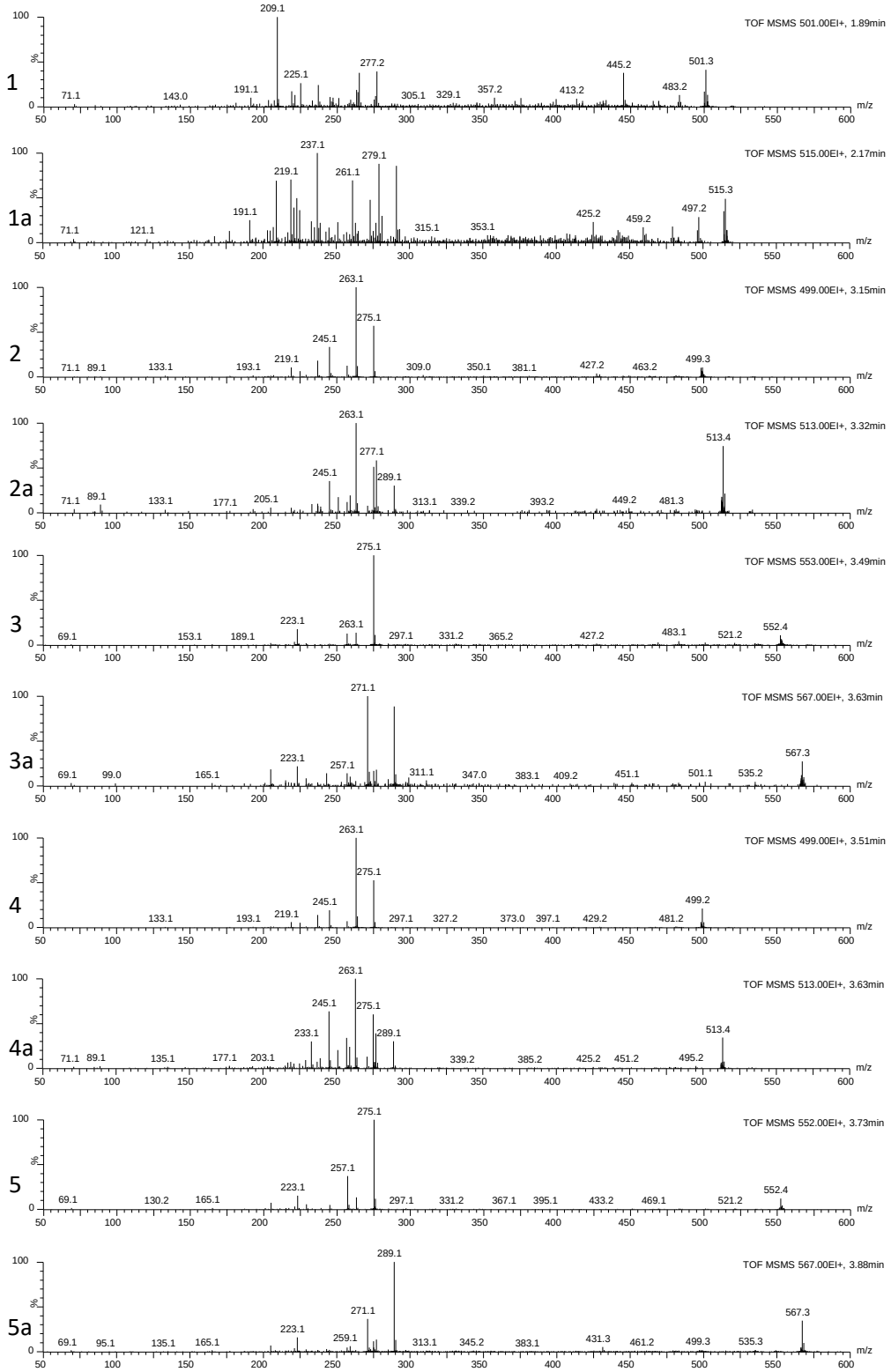


Fig. S7 MS/MS spectra of compound **1** – **5**, higher homologues **1a** – **5a**, and some mayor (**a** - **f**) and minor (**u** - **z**) common components in the *n*-hexane extracts of Peruvian *Hypericum* species

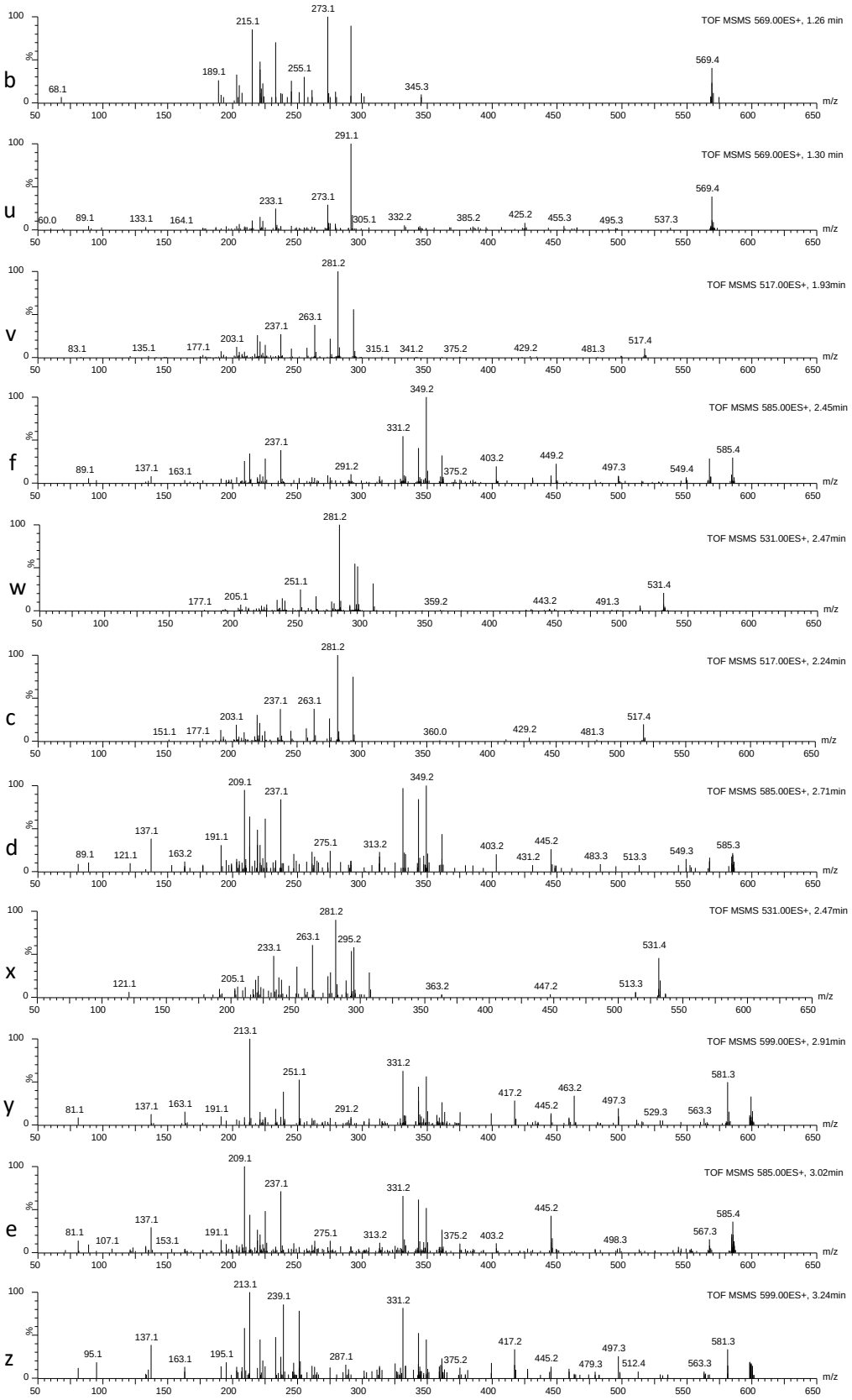


Fig. S7 (Continued)

CONSIDERAÇÕES FINAIS

O gênero *Hypericum* apresenta pouco menos de 500 espécies. Estima-se que cerca de 80 membros do gênero tenham sido estudados fitoquimicamente, os quais tem se mostrado ricos em metabolitos secundários, em especial policetídeos do tipo III que inclui flavonoides, xantonas, naftodiantronas e/ou floroglucinois (HÖLZL e PETERSEN 2003; HILLWIG *et al*., 2008). Os policetídeos do tipo III formam-se da condensação de três moléculas de malonil-CoA e várias substâncias precursoras tais como aminoácidos, derivados de aminoácidos de cadeia ramificada, fenilpropanoides derivados do chiquimato, acido benzoico e acetil-CoA (**Figura C1**). Diversas enzimas policetídeos sintases de tipo III têm evoluído para criar esta grande variedade de policetídeos. Frequentemente estes policetídeos são modificados por outras enzimas, ocorrendo, por exemplo, prenilações como observado na paxantona (xantona) e hiperforina (floroglucinol), entre outras.

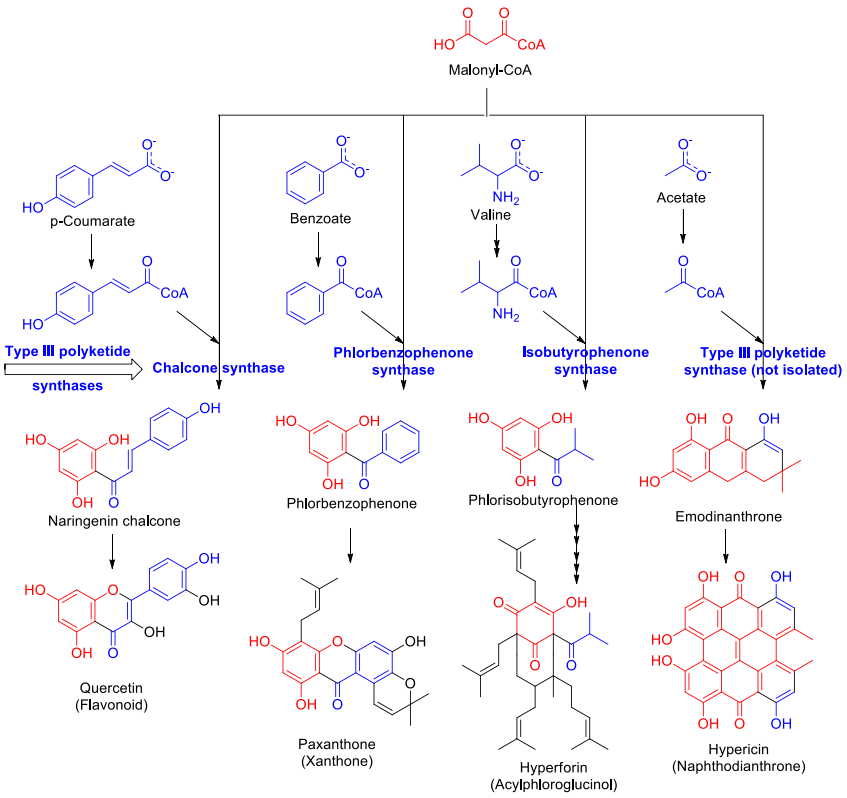


Figura C1. Policetídeos do tipo III e sintases descritas para o gênero *Hypericum*.

A chalcona sintase foi a primeira enzima policetídeos sintases do tipo III a ser caracterizada, sendo a mais conhecida. Os flavonoides observados nas espécies de *Hypericum* como hiperosídeo, rutina, isoquercetrina, quercitrina, quercetina entre outros (**Figura C2**), são produtos derivados da naringenina chalcona (proveniente da atividade enzimática da chalcona sintase). Os resultados do Capítulo II demostram que as espécies Peruanas de *Hypericum*

representam ricas fontes de flavonoides, o que demostra a preservação da rota biossintética destes compostos durante a evolução destas espécies nos Páramos Peruanos. Estes *habitats* são caracterizados por grandes altitudes (superiores a 3000 metros acima do nível do mar) e estão expostos a níveis elevados de radiação UV que pode ter levado a indução da biossíntese de flavonoides nas espécies Peruanas de *Hypericum* pesquisadas. A ocorrência destes metabólitos nas secções taxonômicas e membros do gênero *Hypericum* estão descritos por CROCKETT e ROBSON (2011) e STOJANOVIĆ *et al*., (2013).

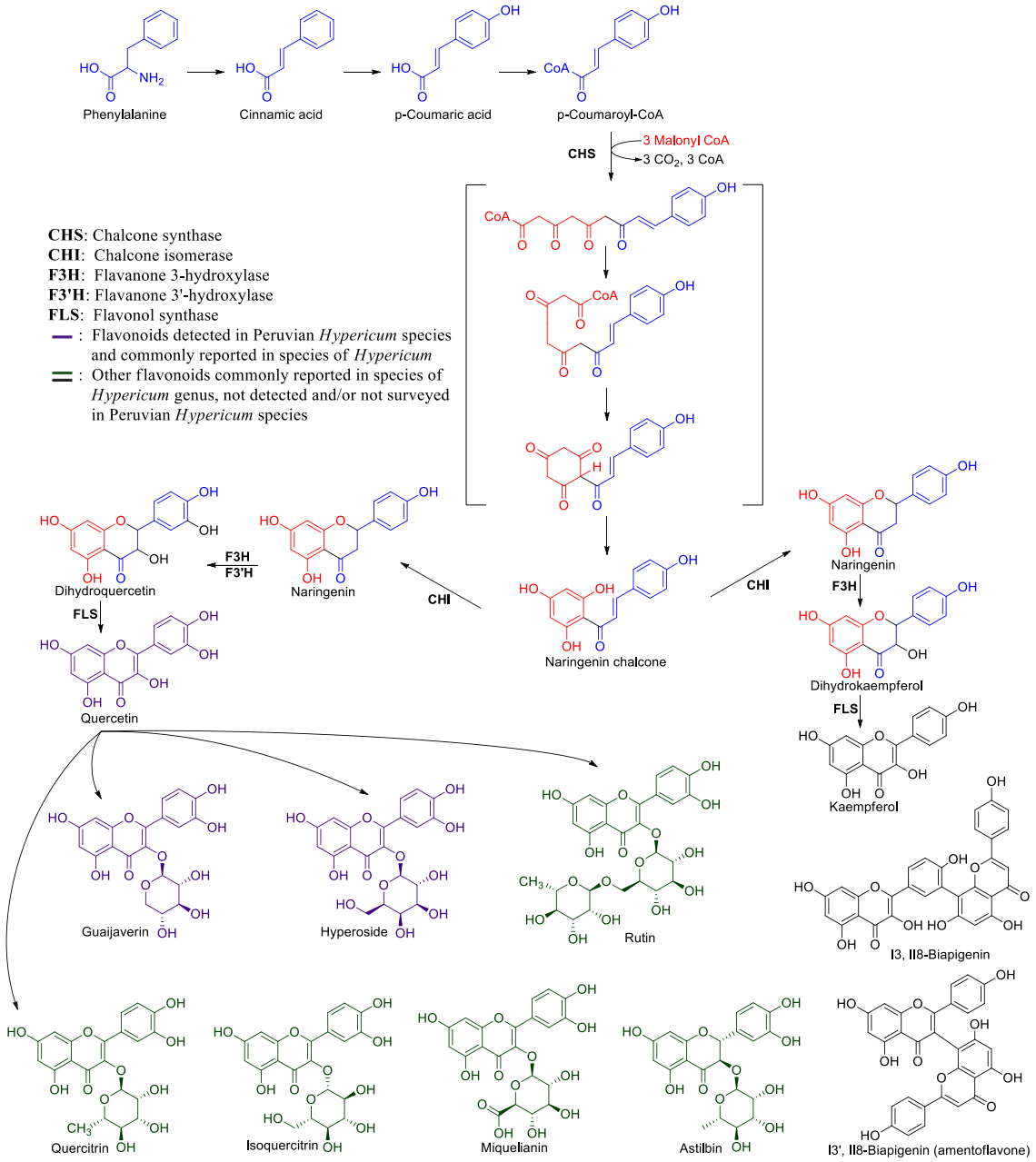


Figura C2. Biossínteses de flavonoides no gênero *Hypericum*.

Biossinteticamente, as xantonas e as benzofenonas têm uma origem comum. A benzofenona sintase é a enzima chave na formação do intermediário 2,4,6-tri-hidroxibenzofenona (**Figura C3**). As vias biossintéticas assim como a ocorrência de xantonas no gênero *Hypericum* são discutidas por DEMIRKIRAN (2007). No presente estudo não foi explorada a ocorrência de xantonas nem benzofenonas nas espécies Peruanas de *Hypericum*. Diversas xantonas têm sido descritas previamente para *H. brasiliense* (ROCHA *et al*., 1994), *H. irazuense* (CROCKETT *et al*., 2010) e *H. laricifolium* (RAMÍREZ-GONZÁILEZ *et al*., 2013), e duas benzofenonas para *H. carinatum* (BERNARDI *et al*., 2005), sugerindo, assim, a preservação da rota biossintética destes compostos nas espécies de *Hypericum* das seções *Brathys* e *Trigynobrathys*.

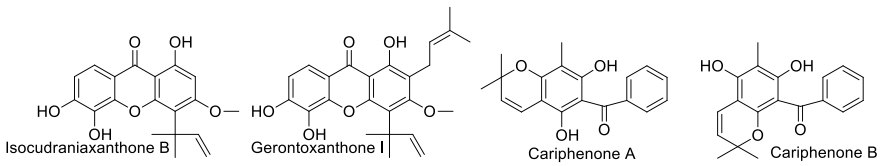
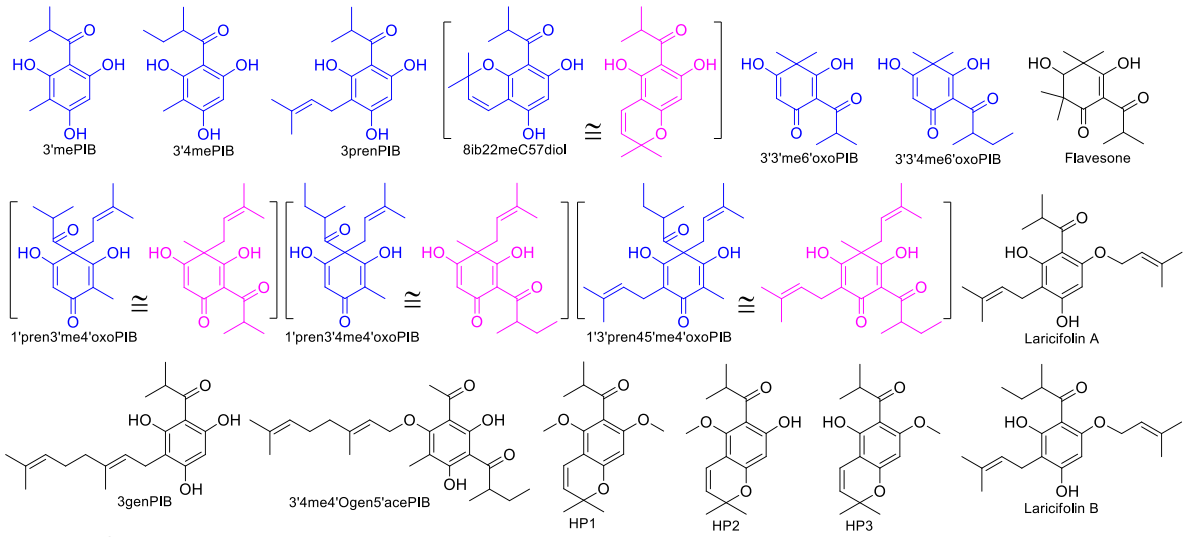


Figura C3. Xantonas e benzofenonas descritas para *H. irazuense* e *H. carinatum*.

Presume-se que a biossíntese das naftodiantronas (hipericina, *pseudo*-hipericina) siga a via dos policetídeos, e, de acordo com essa proposta, num primeiro passo, uma molécula de acetil-CoA condensa-se com sete moléculas de malonil-CoA para formar a emodinantrona. A enzima policetídeo sintase do tipo III, responsável pela biossíntese da emodinantrona, é ainda desconhecida. No entanto, a biossíntese de hipericina a partir da emodinantrona já foi caracterizada (KARIOTI e BILIA, 2010). Ainda, foi verificado que as naftodiantronas são biossintetizadas em células que delimitam um tipo de glândulas denominadas glândulas escuras, demostrando-se, assim, uma correlação positiva entre o tamanho e o número de glândulas escuras e o conteúdo geral de hipericina em *H. perforatum*. No entanto, a presença de glândulas escuras e, consequentemente, naftodiantronas, é comum a 2/3 das espécies do gênero *Hypericum*, característica que tem sido advertida com particular utilidade quimiotaxonômica (CROCKETT e ROBSON, 2011). Os resultados do Capitulo 2 mostram a ausência de hipericinas nas espécies Peruanas de *Hypericum*. A ausência deste tipo de metabolitos em espécies de *Hypericum* das secções *Brathys* e *Trigynobrathys* foi previamente descrita (FERRAZ *et al*., 2002b; CROCKETT e ROBSON, 2011), mostrando que esta via biosintética poderia ter evoluído mais tardiamente. *Brathys* e *Trigynobrathys* são seções

taxonômicas consideradas basais dentro do gênero *Hypericum*, o que poderia explicar a distribuição preferencial de hipericinas nas seções mais evoluídas morfologicamente.

Os compostos derivados do floroglucinol comumente descritos para o gênero *Hypericum* são formados por meio da condensação de varias moléculas de malonil-CoA e derivados de aminoácidos de cadeia ramificada. A florisobutirofenona sintase, uma policetídeo sintase do tipo III, tem sido identificada como a enzima chave na biossíntese de floroglucinois. Os floroglucinois são frequentemente substituídos por prenilas, que posteriormente podem sofrer reações de ciclização dando origem a benzopiranos. Uma grande variedade de floroglucinois tem se descrita para o gênero. No entanto dentro das espécies de *Hypericum* das secções *Brathys* e *Trigynobrathys* é comum o relato de floroglucinois monoméricos e mais frequentemente diméricos. Os resultados do capitulo 2 apresentam o isolamento e elucidação estrutural de dois floroglucinois monoméricos inéditos para *H. laricifolium*. Tendo em conta a presença de três benzopiranos em *H. polyanthemum* mais dois derivados de floroglucinol em *H. japonicum* e um em *H. styphelioides* pode se descrever a ocorrência de oito floroglucinois monoméricos, identificados inequivocamente por RMN 1D e 2D, dentro das espécies de *Hypericum* das seções *Brathys* e *Trigynobrathys* (**Figura C4**).



Observations:
3'mePIB, 3'4mePIB, **3prenPIB**, **8ib22meC57diol**, **3'3'me6'oxoPIB**, **3'3'4me6'oxoPIB**, **1'pre3'me4'oxoPIB**, **1'pren3'4me4'oxoPIB**, **1'3'pren45'me4'oxoPIB**: Blue structures correspond to monomeric acylphloroglucinols identified in *Hypericum gentianoides* by direct infusion Q-TOF-MS analysis of an methanolic extract (Crispin et al. 2013). Pink structures represent possible regioisomers that would not be differentiable by direct infusion Q-TOF-MS analysis from the proposed monomeric acylphloroglucinols if present in plant
Flavesone, and **3'4me4'Ogen5'acePIB**: identified by isolation and NMR in *Hypericum japonicum* (Hu et al. 2000)
3genPIB: identified by isolation and NMR in *Hypericum styphelioides* (Gamiotea-Turro et al. 2004)
HP1, **HP2** and **HP3**: identified by isolation and NMR in *Hypericum polyanthemum* (Ferraz et al. 2001)
Laricifolin A and **laricifolin B**: identified by isolation and NMR in *Hypericum laricifolium* (This study)

Figura C4. Floroglucinois monoméricos reportados para as espécies de *Hypericum* das seções *Brathys* e *Trigynobrathys*.

Recentemente, foi relatada a identificação de nove floroglucinois monoméricos num extrato alcoólico de *H. gentianoides*. Apenas um destes compostos (1'3'pren45'me4'oxoPIB) apresentou concentração suficiente para ser detectável por LC-MS. Os demais oito floroglucinois monoméricos de *H. gentianoides* apresentaram contrações muito baixas, mas que foram detectáveis por meio de análises Q-TOF-MS por infusão direta do extrato. As baixas concentrações destes floroglucinois monoméricos e os altos níveis de floroglucinois diméricos detectados em *H. gentianoides* levaram à formulação da hipótese denominada '*monomer pool concept*' por CRISPIN *et al*. (2013), na qual os floroglucinois monoméricos seriam precursores dos diméricos. Assim, postula-se que os floroglucinois diméricos sejam sintetizados via modificações da unidade básica florisobutirofenona (PIB) para gerar estruturas básicas de monômeros de floroglucinol e de ácido filicínico, os quais posteriormente sofreriam um processo de dimerização para gerar os floroglucinois diméricos.

A enzima responsável pela reação de dimerização dos floroglucinois monoméricos gerando, assim, compostos diméricos é ainda desconhecida, mas o mecanismo da enzima EC 1.21.3.3, responsável pela formação de uma ponte metilenica entre dois anéis aromáticos, é conhecido (KUTCHAN e DITTRICH, 1995). Esta enzima formadora de ponte (BBE, *Berberine brigde enzime*) foi caracterizada em culturas celulares de *Berberis beaniana*, e é responsável pela catalise da ciclização oxidativa da (S)-reticulina, para formar a (S)-scoulerina (**Figura C5 e Figura C6**).

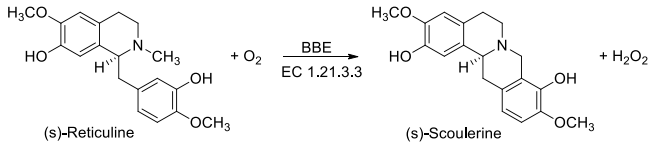


Figura C5. Biossíntese de (S)-scoulerina a partir da (S)-reticulina.

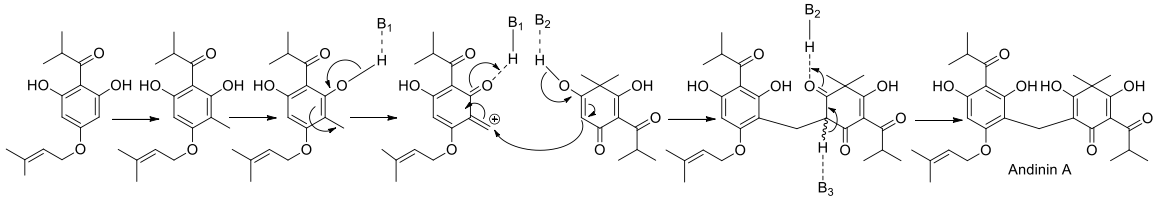
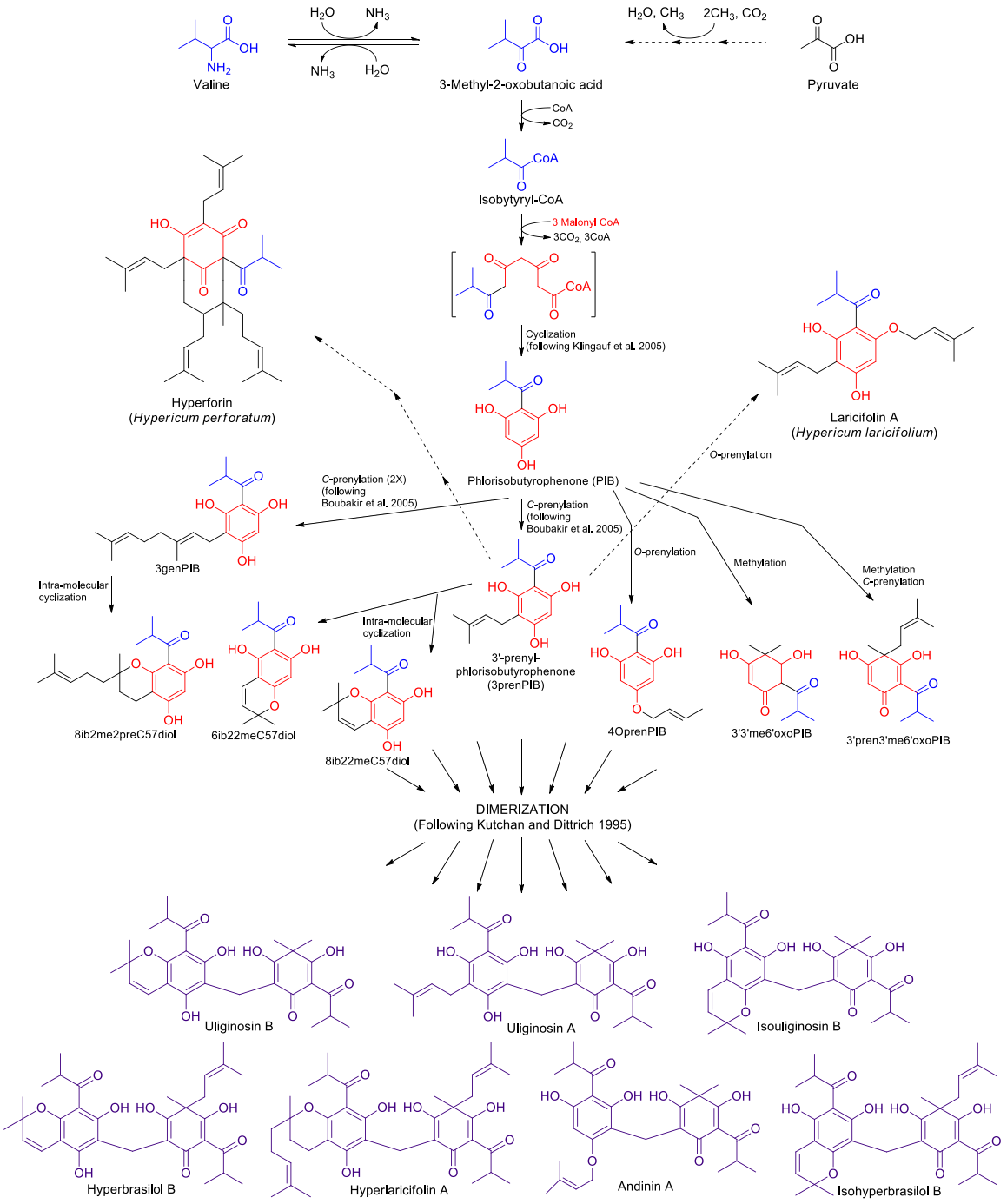
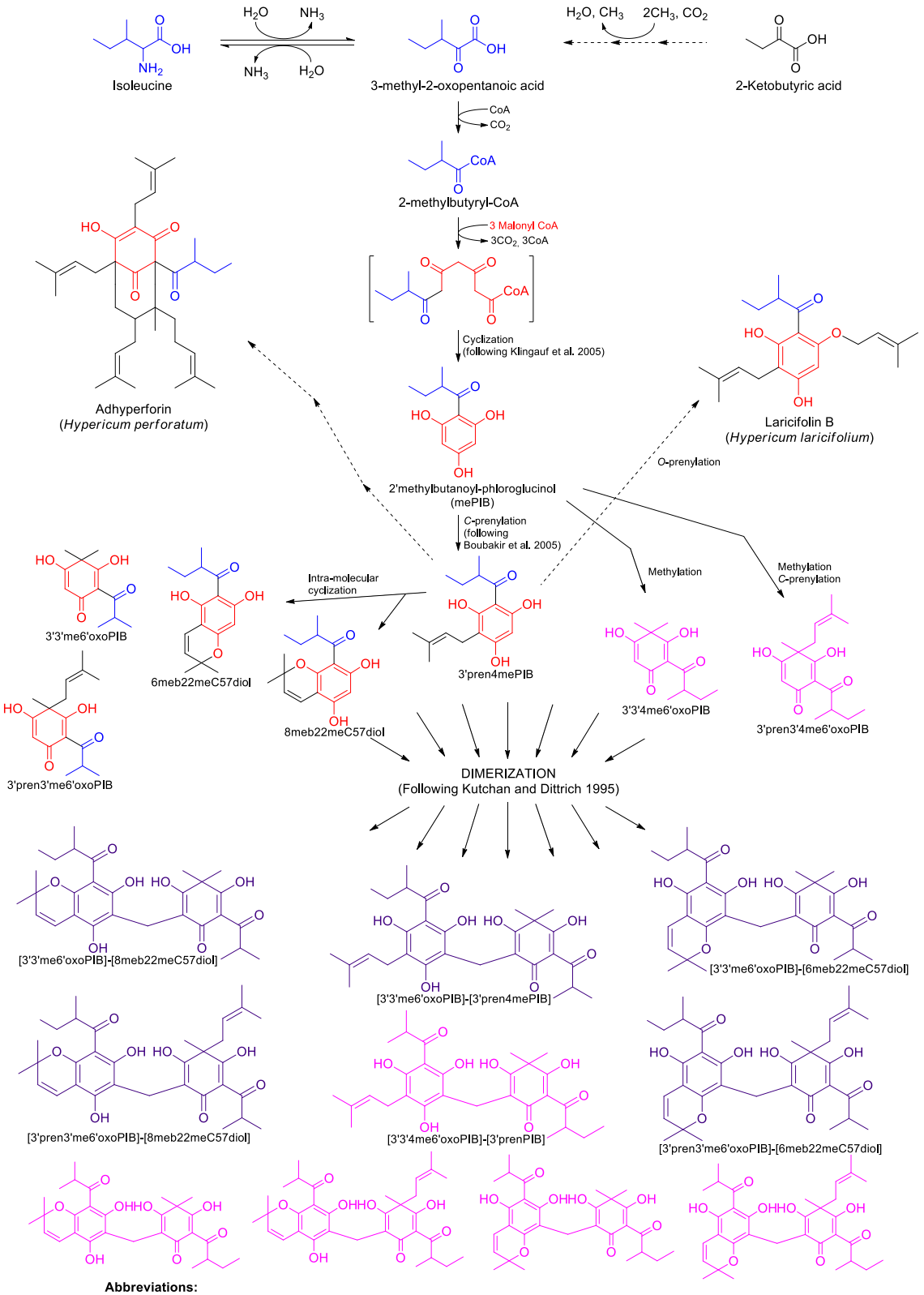


Figura C6. Exemplificação do mecanismo proposto por CRISPIN *et al*., (2013) na formação de ponte metilênica na biossíntese do floroglucinol dimérico andinina A. A formação da ponte metilênica a partir de dois floroglucinois monoméricos prossegue através de um processo multi-passo semelhante ao da reticulina oxidase (KUTCHAN e DITTRICH, 1995). B1, B2 e B3 são resíduos básicos propostos da enzima envolvida na formação dos floroglucinois diméricos.



Abbreviations:
PIB: phlorisobutryophenone (identified by MS in *Hypericum gentianoides*)
3prenPIB: 3'-prenyl-phlorisobutyrophenone (identified by MS in *Hypericum gentianoides*)
3genPIB: 3'-geranyl-phlorisobutyrophenone (identified by NMR in H. *Hypericum styphelioides*)
8ib2me2preC57diol: 8-isobutyryl-2-methyl-2-(prenylmethyl)chromanyl-5,7-diol (identified by NMR in *Hypericum amblycalyx*)
6ib22meC57diol: 6-isobutyryl-2,2-dimethylchromene-5,7-diol (PIB derivative hypothetically proposed)
8ib22meC57diol: 8-isobutyryl-2,2-dimethylchromene-5,7-diol (identified by MS in *Hypericum gentianoides*)
4OprenPIB: 4'-O-prenyl-phlorisobutyrophenone (PIB derivative hypothetically proposed)
3'3'me6'oxoPIB: 3',3'-dimethyl-6'-oxophlorisobutyrophenone (PIB derivative hypothetically proposed)
3'pren3'me6'oxoPIB: 1'-prenyl-3'-methyl-6'-oxophlorisobutyrophenone (PIB derivative hypothetically proposed)
Purple strucutres are acylphloroglucinols identified in Peruvian *Hypericum* species by isolation and NMR

Figura C6. Biossínteses de floroglucinois monoméricos e diméricos nas espécies peruanas de *Hypericum*: derivados da valina



Abbreviations:
mePIB: 2'methylbutanoyl-phloroglucinol (PIB derivative hypothetically proposed)
3'pren4mePIB: 3'-prenyl-2'methylbutanoyl-phloroglucinol (PIB derivative hypothetically proposed)
6meb22meC57diol: 6-2'methylbutanoyl-2,2-dimethylchromene-5,7-diol (PIB derivative hypothetically proposed)
8meb22meC57diol: 8-2'methylbutanoyl-2,2-dimethylchromene-5,7-diol (PIB derivative hypothetically proposed)
3'3'4me6'oxoPIB and **[3'3'4me6'oxoPIB]-[3'prenPIB]** were identified by MS in *H. gentianoides*.
Pink structures are biogenically posible but were not identified in Peruvian *Hypericum* species.
Purple strucutres are acylphloroglucinols identified in Peruvian *Hypericum* species by LC-MS and UPLC-QTOF-MS

Figura C7. (Continuação): derivados da isoleucina

A ocorrência natural de pelo menos 31 floroglucinois diméricos (Capitulo 1) nas espécies de *Hyperium* das seções *Brathys* e *Trignobrathys* sugere a preservação das vias biossintéticas que levam à formação dos floroglucinois diméricos dentro dos membros destas duas seções taxonômicas. A via inicial que leva à formação de floroglucinois monoméricos parece ser comum a todas as espécies do gênero *Hypericum*, mas a via de dimerização poderia ser exclusiva das espécies das seções *Brathys* e *Trigynobrathys*. Tendo em conta os resultados do Capítulo 1, Capitulo 2 e capitulo 4, propõe-se as vias de biossíntese de floroglucinois monoméricos e diméricos nas espécies peruanas de *Hypericum* (**Figura C6 e Figura C7**).

O processo de isolamento por métodos cromatográficos (cromatografia em coluna de sílica e cromatografia planar centrifuga, Cromatroton®) possibilitou a identificação de sete floroglucinois diméricos e dois floroglucinois monoméricos nas frações *n*-hexano de quatro espécies peruanas de *Hypericum* (Capítulo 1 e Capítulo 2). No entanto a análise dos extratos *n*-hexano de seis espécies peruanas de *Hypericum* por meio de uma nova metodologia de HPLC (Capítulo 4) revelou que cada um destes extratos apresenta uma alta complexidade química. Por meio de combinação de técnicas cromatográficas (HPLC-DAD, LC-MS, UPLC-QTOF-MS) foi possível a identificação de mais cinco floroglucinois diméricos adicionais homólogos M + 14, junto com pelo menos 10 compostos tentativamente identificados, que apresentaram um perfil UV e MS indicativo de floroglucinois diméricos. Estes achados suportam a hipótese de que os extratos *n*-hexano das espécies de *Hypericum* das seções *Brathys* e *Trigynobrathys* são fontes copiosas de floroglucinois diméricos.

Extratos e frações de espécies de *Hypericum* têm mostrado uma ampla gama de propriedades farmacológicas destacando-se as propriedades anti-inflamatórias, analgésicas, antidepressivas e antibacterianas. Extratos alcoólicos estandardizados de *H. perforatum* são amplamente utilizados no tratamento de depressão leve a moderada (STEVINSON e ERNST, 2003). Estes extratos contem uma ampla gama de constituintes sendo os principais, flavonoides, naftodiantronas e floroglucinois, tendo se atribuído a estes o efeito observado (HÖLZL e PETERSEN, 2003). A observação de que muitas outras espécies de *Hypericum* que não produzem naftodiantronas nem floroglucinois do tipo da hiperforina, mas que são ricas em flavonoides destaca a importância destes compostos na atividade antidepressiva de extratos de *Hypericum*. Os resultados do capítulo 3 mostra que os extratos alcoólicos de quatro espécies de *Hypericum* peruanas apresentam potencial atividade antidepressiva. A

análise fitoquímica destes extratos mostrou uma alta concentração de flavonoides em especial hiperosídeo, e uma baixa concentração de floroglucinois diméricos, pelo que os flavonoides destacam-se como os principais responsáveis do efeito observado.

Frações lipofílicas de *H. caprifoliatum* e *H. polyanthemum* também têm mostrado potencial atividade antidepressiva. O efeito destas frações tem sido atribuído à presença de floroglucinois diméricos, como HC1 e uliginosina B (STEIN *et al*., 2012). A uliginosina B tem mostrado um perfil promissor de atividade antidepressiva mostrando que floroglucinois diméricos poderiam ser moléculas protótipo para o desenvolvimento de novos antidepressivos. Os resultados do Capítulo 2 mostram que a andinina A, floroglucinol dimérico inédito isolado das raízes de *H. andinum*, apresenta potencial atividade antidepressiva.

Extratos e frações de *H. gentianoides* têm mostrado capacidade de bloquear a produção de mediadores pro-inflamatórios, prostaglandina E_2 e óxido nítrico, induzida por LPS, usando macrófagos murinhos RAW 264.7 como modelo experimental. A uliginosina A foi identificada como um dos componentes reesposáveis por está atividade (HILLWIG *et al*., 2008; HUANG *et al*., 2011). Os resultados do capitulo 4 mostram que as frações *n*-hexano de seis espécies de *Hypericum* são misturas complexas de floroglucinois diméricos. Estes extratos inibiram a quimiotaxia induzida por LPS em neutrófilos polimorfonucleares, mostrando potencial atividade anti-inflamatória. O componente principal destes extratos, a uliginosina B, também mostrou um perfil de atividade anti-inflamatória dose dependente no modelo testado.

Assim o presente estudo apresenta as espécies peruanas de *Hypericum* como fontes ricas de flavonoides derivados da quercetina e floroglucinois diméricos com potencias propriedades anti-inflamatórias e antidepressivas.

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