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Fürer, Karin ; Raith, Melanie ; Brenneisen, Rudolf ; Mennet, Monica ; Simões-Wüst, Ana Paula ; von Mandach, Ursula ; Hamburger, Matthias ; Potterat, Olivier

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Original paper

Two new flavonol glycosides and a metabolite profile of *Bryophyllum pinnatum*, a phytotherapeutic used in obstetrics and gynaecology

Karin Fürer[1,2], Melanie Raith[1], Rudolf Brenneisen[3], Monica Mennet[4], Ana Paula Simões-Wüst[5], Ursula von Mandach[2], Matthias Hamburger[1], and Olivier Potterat[1*]

Affiliation

[1] Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, 4056 Basel, Switzerland

[2] Department of Obstetrics, University Hospital Zurich, 8091 Zürich, Switzerland

[3] Department of Clinical Research, University of Bern, 3010 Bern, Switzerland

[4] Weleda AG, 4144 Arlesheim, Switzerland

[5] Research Department, Paracelsus Hospital Richterswil, 8805 Richterswil, Switzerland

Correspondence

PD Dr. Olivier Potterat, Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstraße 50, CH-4056 Basel, Switzerland. Email: Olivier.Potterat@unibas.ch Phone: +41 61 267 15 34 Fax: +41 61 267 14 74

Abstract

Bryophyllum pinnatum is a succulent perennial plant native to Madagascar which is used in anthroposophical medicine to treat psychiatric disorders, and as a tocolytic agent to prevent premature labour. We performed a metabolite profiling study in order to obtain a comprehensive picture of the constituents in *B. pinnatum* leaves, and to identify chromatographic markers for quality control and safety assessment of medicinal preparations. Preliminary HPLC-PDA-ESIMS analyses revealed that flavonoid glycosides were the main UV-absorbing constituents in the MeOH extract of *B. pinnatum*. Two phenolic glucosides, syringic acid *β*-D-glucopyranosyl ester (**1**) and 4'-O-*β*-D-glucopyranosyl-*cis*-p-coumaric acid (**2**), as well as nine flavonoids (**3**-**11**) including kaempferol, quercetin, myricetin, acacetin, and diosmetin glycosides were unambiguously identified by ^{1}H and 2D NMR analysis after isolation from a MeOH extract. The flavonol glycosides quercetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside 7-O-*β*-D-glucopyranoside (**3**) and myricetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside (**4**) were new natural products. With the aid of HPLC-PDA-APCIMS and authentic references isolated from the related species *B. daigremontianum*, the presence of four bufadienolides, bersaldegenin-1-acetate (**12**), bryophyllin A (**13**), bersaldegenin-3-acetate (**14**), and bersaldegenin-1,3,5-orthoacetate (**15**) was detected in *B. pinnatum*.

Key words

Bryophyllum pinnatum, *Kalanchoe pinnata*, Crassulaceae, HPLC-PDA-MS profiling, flavonoids, bufadienolides

Abbreviations:

APCI: Atmospheric-pressure chemical ionization

ESI: Electrospray ionization

PDA: Photodiode array

Introduction

Bryophyllum pinnatum (Lam.) Oken (syn. *Kalanchoe pinnata* Pers., *Bryophyllum calycinum* Salisb.) is a succulent perennial plant native to Madagascar and belongs to the family of Crassulaceae. It is commonly known as Life plant, Air plant, Love plant, and Goethe plant. *B. pinnatum* has been widely used in traditional medicine, especially in Madagascar, Indonesia, India, Nigeria, Trinidad and Tobago where the leaves have been utilized to treat jaundice [1], skin diseases, urinary problems, hypertension, and for its cooling properties in topical use [2-4].

In 1921, *B. pinnatum* preparations were established by Rudolf Steiner as anthroposophical medicines to treat hysteria [5]. Later, *B. pinnatum* was also used in obstetrics and gynaecology as a tocolytic agent to prevent premature labour [6] and, more recently, to treat sleep disorders in pregnancy. As a tocolytic agent *B. pinnatum* showed only mild and few adverse effects and was very well tolerated [7]. We could show that the leaf juice inhibits oxytocin-induced increase of intracellular calcium concentration in human myometrial cells [8] and induces myometrial relaxation in vitro [9]. Recently, we reported that *B. pinnatum* leaf press juice also inhibits porcine detrusor contractility *in vitro* [10]. To explore the potential of *B. pinnatum* as a treatment for patients suffering from overactive bladder syndrome, a pilot study in humans was performed. A positive trend for a *B. pinnatum* preparation compared to placebo could be shown [11].

With respect to pharmacological properties of *B. pinnatum,* antileishmanial [12], antiulcer [13], antibacterial [14,15], antitumor promoting [16], immunosuppressive [17], and antihypertensive effects [18] have been reported. Compounds identified in the plant include flavonoids, triterpenes, phytosterols, bufadienolides, fatty acids, and minerals [19]. The flavonoid fraction was found to consist mainly of kaempferol and quercetin glycosides, some of which have shown *in vitro* antileishmanial activity [12,20]. A series of bufadienolides such as bryophyllins A - C, and bersaldegenin derivatives have been isolated [19,21]. These compounds reportedly possess sedative and positive inotropic properties, as well as central nervous system (CNS) related activities [22]. For safety assessment and quality control of phytomedicines containing *B. pinnatum*, detailed information on the metabolite profile of is required. There have been only few relevant analytical studies on *B. pinnatum*. Four flavonoids were assigned in the HPLC-UV-MS chromatogram of an aqueous extract [23]. A series of flavonoids were also identified by HPLC-UV in a chromatographic fraction. [24]. No study includes, however, a comprehensive analysis of the constituents of the plant. We therefore conducted a metabolite profiling of the MeOH extract in order to identify useful chromatographic markers for quality control and safety assessment, whereby special emphasis was put on flavonoids and bufadienolides.

Results and Discussion

To obtain a comprehensive metabolite profile, leaves of *B. pinnatum* were extracted with MeOH, and the extract submitted to HPLC-PDA-ESIMS analysis (**Fig. 1**). The UV trace and PDA spectra revealed the presence of several peaks of UV-absorbing phenolic compounds. In addition, a group of large peaks eluting between 19 and 24 min were detected in the MS trace. By comparison with reference compounds previously isolated by the authors from *Lycium barbarum* (unpublished results), two peaks at t_R 22.0 min (*m/z* 520.6 $[M]^+$) and t_R 23.2 min (*m/z* 496.6 $[M]^+$) were assigned to linoleoyl lysophosphatidylcholine, and palmitoyl lysophosphatidylcholine, respectively. In addition, a peak at t_R 20.4 min (*m/z* 518.6) was

tentatively assigned to α-linolenoyl lysophosphatidylcholine. Most peaks detected in the UV trace could be identified after targeted purification by a combination of Sephadex LH-20 and Diaion HP-20 column chromatography, and preparative and semi-preparative HPLC. The structures were established on the basis of UV, ^{1}H and ^{13}C NMR spectra, and by comparison with literature data.

Compounds **1** (*m/z* 743.2 $[2M + H]^+$) and **2** (*m/z* 651.2 $[2M - H]^-$, 325.2 $[M - H]^-$) were shown to be phenolic acid derivatives. They were identified as syringic acid *β*-D-glucopyranosyl ester (**1**) [25] and 4'-O-*β*-D-glucopyranosyl-*cis*-p-coumaric acid (**2**) [26]. The *cis*-configuration of the olefinic double bond in **2** is supported by the $^{3}J_{HH}$-coupling constant of the corresponding protons (H-2 δ_H 5.85 ppm, H-3 δ_H 6.53 ppm, J = 12 Hz).

Compounds **3-11** exhibited UV spectra characteristic for flavonoids. Their structures (**Fig. 2**) were assigned by ESIMS, NMR, and by comparison with literature data. Compounds **6** (*m/z* 581.3 $[M + H]^+$, 449.2 $[(M + H) - 132]^+$, 303.2 $[(M + H) - 132 - 146]^+$) and **8** (*m/z* 449.2 $[M + H]^+$, 303.2 $[(M + H) - 146]^+$) were quercetin glycosides. They were identified as quercetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside (**6**) [12], and quercitrin (quercetin 3-O-*α*-L-rhamnopyranoside, **8**) [20], respectively. Compound **5** (*m/z* 465.1 $[M + H]^+$, 319.2 $[(M + H) - 146]^+$) was identified as myricitrin (myricetin 3-O-*α*-L-rhamnopyranoside, **5**) [27]. Compounds **9** (*m/z* 565.3 $[M + H]^+$, 433.2 $[(M + H) - 132]^+$, 287.2 $[(M + H) - 132 - 146]^+$) and **10** (*m/z* 565.3 $[M + H]^+$, 433.2 $[(M + H) - 132]^+$, 287.2 $[(M + H) - 132 - 146]^+$) were shown to be kaempferol glycosides, namely kaempferol 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside (**9**) [12] and kaempferol 3-O-*β*-D-xylopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside (**10**) [28], respectively. Compounds **7** (*m/z* 609.4 $[M + H]^+$) and **11** (*m/z* 593.4 $[M + H]^+$) were identified as diosmine (diosmetin 7-*O*-*α*-L-rhamnopyranosyl-(1 → 6)-*β*-D-glucopyranoside, **7**) [29] and acacetin 7-O-*α*-L-rhamnopyranosyl-(1 → 6)-*β*-D-glucopyranoside (**11**) [30], respectively. The position of the sugar residues and the interglycosidic linkages in all compounds were supported by HMBC correlations. ^{1}H and ^{13}C NMR data are provided as Supporting Information.

Compounds **3** and **4** are new flavonol glycosides. Their structures were established as follows: The HRESIMS spectrum of **3** showed a quasimolecular $[M + Na]^+$ ion peak at *m/z* 765.1844, in agreement with a molecular formula of $C_{32}H_{38}O_{20}$. Fragment ions were detected at *m/z* 611.3 $[(M + H) - 132]^+$, and 465.2 $[(M + H) - 132 - 146]^+$. The aglycone was identified as quercetin from its NMR data (**Table 1**) and by comparison with compounds **6** and **8**. Acid hydrolysis afforded D-glucose, L-rhamnose and L-arabinose. The monosaccharides were identified by GC-MS analysis after derivatization with L-cysteine methyl ester and silylation. The *α*-configuration of the arabinopyranosyl, and the β-configuration of the glucopyranosyl residues were derived from the coupling constant of the anomeric protons at δ_H 4.16 (d, 5.9 Hz, H-1''') and δ_H 5.06 ppm (d, 6.9 Hz, H-1''''), respectively. The α-configuration of the rhamnopyranosyl residue was assigned by ^{13}C NMR [12]. The NMR data of the disaccharide moiety were

in full agreement with those recorded for compound **6**. The interglycosidic linkage was confirmed by an HMBC correlation between H-1''' (δ_H 4.16 ppm) of the α-L-arabinopyranosyl moiety and C-2'' (δ_C 80.1) of the α-L-rhamnopyranosyl residue. The HMBC correlation between H-1'' of the rhamnosyl moiety (δ_H 5.36) and C-3 of the aglycone (δ_C 135.0) revealed the attachment of the disaccharide moiety. The attachment of the β-D-glucopyranosyl moiety at C-7 was established by NOESY contacts of the anomeric proton H-1'''' with H-6 and H-8. The structure of **3** was thus established as quercetin 3-O-α-L-arabinopyranosyl-(1 $\rightarrow$ 2)-α-L-rhamnopyranoside 7-O-β-D-glucopyranoside.

A molecular formula of $C_{26}H_{28}O_{16}$ for compound **4** was established by HRESIMS spectroscopy ($[M + Na]^+$ quasimolecular ion at *m/z* 619.1290). Fragment ions were observed at *m/z* 465.2 $[(M + H) - 132]^+$, and 319.2 $[(M + H) - 132 - 146]^+$ in the ESIMS spectrum. The NMR data (**Table 2**) of the disaccharide moiety were almost identical with those of compounds **3** and **6**. An HMBC correlation between H-1'' of rhamnose (δ_H 5.23) and C-3 of the aglycone (δ_C 135.0) indicated the attachment of the sugar moiety. Compound **4** differed from **6** only in the substitution of the B-ring. A signal corresponding to two protons appeared as singulet at δ_H 6.93 ppm. The NMR data of the aglycone were in full agreement with those of compound **5**, confirming the aglycone to be myricetin. Compound **4** was thus myricetin 3-O-α-L-arabinopyranosyl-(1 $\rightarrow$ 2)-α-L-rhamnopyranoside. Among the isolated compounds, only the quercetin glycosides **6** and **8**, as well as the kaempferol glycoside **9** were previously identified in *B. pinnatum* [12].

Occurrence of bufadienolides in *B. pinnatum* has been reported [31], but we could not detect them by HPLC-PDA-ESIMS analysis of the MeOH extract. For targeted chromatographic detection, reference compounds were isolated from a related species, *B. daigremontianum*, known to contain higher concentrations of bufadienolides. Compounds **12-15** were obtained from the CH_2Cl_2-soluble fraction of the MeOH extract by a combination of preparative and semi-preparative HPLC on RP-18. They were identified as bersaldegenin-1-acetate (**12**, *m/z* 475.4 $[M + H]^+$) [22], bryophyllin A (**13,** *m/z* 473.4 $[M + H]^+$) [32], bersaldegenin-3-acetate (**14,** *m/z* 475.5 $[M + H]^+$) [22], and bersaldegenin-1,3,5-orthoacetate (**15,** *m/z* 457.3 $[M + H]^+$) [22] by APCIMS, ^{1}H and 2D NMR. ^{1}H and ^{13}C NMR data of **12-15** are provided as Supporting Information, and their structures are shown in **Fig. 3**.

Using the reference compounds **12-15** isolated from *B. daigremontianum*, the presence of bufadienolides in *B. pinnatum* was confirmed by HPLC-APCIMS. The four bufadienolides **12-15** could be detected in the CH_2Cl_2-soluble fraction of the MeOH extract of *B. pinnatum* (**Fig. 4**). To the best of our knowledge, bersaldegenin-1-acetate (**12**) had not been previously reported as constituent of *B. pinnatum*, while compounds **13-15** were already described [33]. It is noteworthy that bufadienolides **12-15** could not be detected by HPLC-ESIMS in positive or negative ion mode under these conditions.

In conclusion, our study provides a detailed metabolite profile of the leaves of *B. pinnatum*. Two phenolic acids, several flavonol and O-methylated flavone glycosides, a bufadienolide, and

lysophosphatidylcholine derivatives were identified for the first time in this plant which is currently the object of clinical investigations in different therapeutic indications [10,11]. Two flavonol glycosides were new natural products. Most peaks detected by HPLC-PDA-ESIMS could be structurally assigned. In addition, bufadienolides could be unambiguously detected by HPLC-APCIMS. In agreement with previous reports, flavonoids are the main UV-active constituents of the MeOH leaf extract. The major peak was found to be quercetin 3-O-α-L-arabinopyranosyl-(1 $\rightarrow$ 2)-α-L-rhamnopyranoside (**6**), confirming a previous report of its occurence as the main flavonoid in an aqueous extract [23]. The flavonoids identified are suited as chemical markers for quality control of medicinal preparations, whereas the bufadienolides are important for safety assessment regarding the presence or absence of potentially toxic constituents in specific products.

Material and methods

General experimental procedures

Solvents were from Scharlau. Technical grade solvents were used after redistillation for extraction and column chromatography (CC). HPLC grade solvents were used for HPLC. HPLC grade water was obtained by an EASY-pure II (Barnstead) water purification system. Diaion HP-20 (250 µm) was purchased from Supelco. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals. A pump (model 881, Büchi) and a fraction collector (Superfrac, Pharmacia Biotech) were used for CC on Sephadex LH-20. For TLC analysis, silica gel plates F_{254} (10-12 µm; Merck) were used with MeOH/EtOAc (1:3) or $CHCl_3$/MeOH/H_2O (65:35:5) as mobile phase. Detection was at UV 254 and 366 nm, and after staining with vanillin/sulfuric acid reagent or Natural Product Reagent A (1% ethanolamine diphenylborate, Sigma-Aldrich). Silica gel plates 60_{F264s} (5-6 µm; Merck) were used for HPTLC with EtOAc/HCOOH/AcOH/H_2O (100:11:11:26) as mobile phase. Detection was at 366 nm after spraying with Natural Product Reagent A. GC-MS analysis was performed using a HP 5890 Series II gas chromatograph equipped with a HP 5971 mass selective detector (Hewlett Packard). HPLC-PDA-MS analyses were performed using an Agilent 1100 Series HPLC coupled to a Bruker Esquire 3000 plus mass spectrometer. Separations were performed on a C_{18} SunFire^{TM} column (3.5 µm, 3 x 150 mm, Waters) equipped with a guard column (3 x 10 mm). The samples were dissolved in DMSO at a concentration of 3 mg/mL (extract, fractions) or 0.6 mg/mL (pure compounds). 10 µL (fractions, compounds) or 20 µL (extract) were injected. The mobile phase consisted of 0.1% aqueous formic acid (A) and MeCN (B) and a linear gradient of 5-100% B in 30 min was applied. The flow rate was 0.5 mL/min. UV spectra were recorded from 210 to 400 nm. ESIMS spectra were obtained in positive and negative ion modes between

m/z 200 and 1500. APCIMS spectra were recorded in the positive ion mode between *m/z* 150 and 1500. Semi-preparative HPLC was carried out on an Agilent 1100 Series system connected to a PDA detector. Separations were performed on a Waters SunFire^{TM} C_{18} column (5 µm, 10 x 150 mm) equipped with a precolumn (5 µm, 10 x 10 mm). The mobile phase consisted of 0.1% aqueous formic acid (A) and MeCN (B). The flow rate was 3 mL/min. Preparative HPLC was performed on a Shimadzu LC-8A instrument connected to a SPD-M10AVP PDA detector. A Waters SunFire^{TM} C_{18} OBD^{TM} column (5 µm, 30 x 150 mm) was used for separation. The mobile phase consisted of 0.1% aqueous formic acid (A) and MeCN (B). The flow rate was 20 mL/min. ^{1}H NMR and 2D NMR (COSY, HSQC, HMBC, selective TOCSY, HSQC-TOCSY) data were recorded in DMSO-d_6 or in $CDCl_3$ on a Bruker Avance III^{TM} 500 MHz NMR spectrometer equipped with a 1 mm TXI microprobe. Data were processed with Topspin 2.1 software (Bruker). Optical rotation was measured on a Perkin Elmer Model 341 polarimeter. UV spectra of **3** and **4** were recorded on a Lambda 35 spectrophotometer (Perkin Elmer). HRESIMS data were obtained on a MicrOTOF mass spectrometer (Bruker Daltonics).

Plant material

Bryophyllum pinnatum leaves were harvested from plants cultivated in Schwäbisch Gmünd, Germany, by Weleda Schwäbisch Gmünd, Germany, in July and August 2010. A voucher specimen (ZSS 29715) has been deposited at The Zurich Succulent Plant Collection. *Bryophyllum daigremontianum* leaves were harvested from plants grown in Arlesheim, Switzerland by Ita Wegman Hospital Arlesheim, Switzerland in September 2011. A voucher specimen (838) has been deposited at the Division of Pharmaceutical Biology, University of Basel. After harvesting, the leaves were frozen and stored at -20°C until processing.

Extraction

The frozen leaves of *B. pinnatum* and *B. daigremontianum* were lyophilized. The dried leaves were pulverized in a mortar, and the powder (*B. pinnatum*: 593.4 g, *B. daigremontianum*: 37.5 g) was extracted with MeOH (*B. pinnatum*: 6 L, *B. daigremontianum*: 400 mL). The suspension was stirred for 2 h and subsequently sonicated for additional 20 min. The extract was filtered and evaporated under reduced pressure to yield the MeOH extract (*B. pinnatum*: 53.4 g; *B. daigremontianum*: 7.1 g).

Fractionation of *B. pinnatum* leaf extract

A portion of the MeOH extract (9.0 g) was dissolved in 20 mL of MeOH, applied to a Sephadex LH-20 column (5.5 x 100 cm i.d.) and eluted with MeOH at a flow rate of 2 mL/min. 9-min fractions were collected and combined based on TLC analysis to afford 10 main fractions: B1 (Fr. 1-51, 0.04 g), B2 (Fr.

52-67, 0.38 g), B3 (Fr. 68-79, 2.68 g), B4 (Fr. 80-97, 2.23 g), B5 (98-110, 0.17 g), B6 (111-123, 0.42 g), B7 (Fr. 124-146, 0.16 g), B8 (Fr. 147-165, 0.33 g), B9 (Fr. 166-196, 0.05 g), and B10 (Fr. 197-241, 0.35 g). Based on HPTLC and HPLC-UV-ESIMS analyses, fractions B4, B6, and B8 were selected for further investigation. Fraction B4 (2.21 g) was separated by CC (2.5 x 41 cm i.d.) on Diaion HP-20. The sample was dissolved in H_2O, and the column eluted successively with 750 mL of H_2O and 1 L of MeOH. An aliquot (610 mg) of the MeOH fraction eluted from fraction B4 was separated by preparative HPLC using a linear gradient of 5-45% B in 30 min. The sample dissolved in DMSO (1 g/mL) was injected as 6 aliquots to provide compounds **1** (3.6 mg, t_R 11.5 min), **11** (5.5 mg, t_R 25.0 min) and a mixture (3.4 mg, t_R 13.6 min) which was further separated by semi-preparative HPLC with a linear gradient of 10-30% B in 30 min to provide compounds **2** (0.9 mg, t_R 10.7 min,) and **3** (1.1 mg, t_R 11.4 min). A second aliquot (506 mg) of the same fraction was separated by semi-preparative HPLC with a linear gradient of 10-45% B in 30 min. The sample dissolved in 1 mL DMSO was injected in 11 portions to yield compound **7** (4.9 mg, t_R 14.3 min). Fraction B6 (390 mg) was separated by preparative HPLC with a linear gradient of 20-50% B for 30 min. The sample dissolved in DMSO (125 mg/mL) was injected as 3 aliquots to give compounds **4** (2.4 mg, t_R 8.2 min), **6** (71.6 mg, t_R 10.0 min), **9** (11.0 mg, t_R 12.0 min), and **10** (1.0 mg, t_R 12.6 min). Fraction B8 (430 mg) was separated by preparative HPLC as 3 aliquots using the same system to result in compounds **5** (5.1 mg, t_R 9.0 min), and **8** (2.1 mg, t_R 11.4 min).

Quercetin 3-O-α-L-arabinopyranosyl-(1 → 2)-α-L-rhamnopyranoside 7-O-β-D-glucopyranoside (**3**): yellow amorphous powder. UV (MeOH): λ_{max} (log ε): 207 (4.55), 256 (4.27), 268 (4.16), 350 (4.03); $[\alpha]_D$ - 94 (*c* 0.044, MeOH); ^1H and ^{13}C NMR data (DMSO-d_6): see **Table 1**. HRESIMS: *m/z* 765.1844 $[M + Na]^+$ (calcd. for $C_{32}H_{38}NaO_{20}$: 765.1849); ESIMS: *m/z* 743.2 $[M + H]^+$, 611.3 $[(M + H) - 132]^+$, 465.2 $[(M + H) - 132 - 146]^+$.

Myricetin 3-O-α-L-arabinopyranosyl-(1 → 2)-α-L-rhamnopyranoside (**4**): yellow amorphous powder. UV (MeOH): λ_{max} (log ε): 209 (4.41), 258 (3.92), 303 (sh, 3.55), 353 (3.81); $[\alpha]_D$ - 72 (*c* 0.069 MeOH); ^1H and ^{13}C NMR data (DMSO-d_6): see **Table 2**. HRESIMS: *m/z* 619.1290 $[M + Na]^+$ (calcd. for $C_{26}H_{28}NaO_{16}$: 619.1270); ESIMS: *m/z* 597.1 $[M + H]^+$, 465.2 $[(M + H) - 132]^+$, 319.2 $[(M + H) - 132 - 146]^+$.

Acid hydrolysis and sugar analysis

Compound **3** (0.5 mg) was heated at 100 °C in 2N HCl (1 mL) for 2 h. After cooling, the mixture was extracted with EtOAc (2 x 0.5 mL), and the aqueous phase freeze-dried. The sugars were redissolved in anhydrous pyridine, derivatized with L-cysteine methyl ester hydrochloride (200 µL, 60 ºC, 1 h) and subsequently silylated with hexamethyldisilazane and chlorotrimethylsilane (Fluka) in pyridine (2:1:10; 300 µL; 60 ºC, 30 min). GC-MS analysis was performed on a DB-225MS column (0.25 µm; 0.25 mm x

30 m; Agilent). The oven temperature was initially held 2 min at 150 °C, then increased to 270 °C at a rate of 5 °C/min, and finally kept at 240 °C for 10 min. The injector temperature was 300 °C and the transfer line temperature 280 °C. The He pressure was 0.8 bar and the splitting ratio 1:10. L-arabinose (t_R 13.93 min), L- rhamnose (t_R 14.62 min) and D-glucose (t_R 16.23 min) were identified by comparison with reference sugars treated under the same conditions.

Isolation of bufadienolides

A portion (4.0 g) of the MeOH extract of *B. daigremontianum* was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 soluble fraction (0.9 g) was shown by HPLC-PDA-APCIMS analysis to contain the bufadienolides, and was separated by preparative HPLC using a linear gradient of 5-100% B for 30 min. The sample was dissolved in 4.4 mL DMSO and injected as 11 aliquots. Compounds **12** (2.2 mg, t_R 13.3 min) and **15** (4.1 mg, t_R 17.8 min), and a bufadienolide mixture (1.5 mg, t_R 13.9 min) were obtained. The latter was further separated by semi-preparative HPLC using a linear gradient of 10-80% B in 30 min. Compounds **13** (0.7 mg, t_R 13.9 min) and **14** (0.8 mg, t_R 14.2 min) were obtained.

Detection of bufadienolides in *B. pinnatum*

A portion of the MeOH extract (1.0 g) of *B. pinnatum* was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 soluble fraction (80 mg) was analyzed by HPLC-PDA-APCIMS. The sample was dissolved in DMSO (1 mg/mL) and 20 µL was injected. Analysis was performed with a linear gradient of 5-100% B in 30 min, at a flow rate of 0.5 mL/min. Bufadienolides **12**-**15** were identified by comparison with reference compounds isolated from *B. daigremontianum*.

Supporting information

^{1}H NMR spectra of **3** and **4**, as well as ^{1}H and ^{13}C NMR data of compounds **1, 2**, and **5**-**15** are provided as Supporting Information.

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Conflict of Interest

M.M. is employee of Weleda AG.

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Table 1 ^{1}H and ^{13}C NMR data of compound **3** in DMSO-d_6

position	δ_H (m, *J* in Hz)	δ_C [b),c)]	position[a)]	δ_H (m, *J* in Hz)	δ_C [b)]
Aglycone			*Rha*		
2	-	n.d.	1‘‘	5.36 (s)	100.9
3	-	135.0	2‘‘	4.06 (s)	80.1
4	-	n.d.	3‘‘	3.63 (m)	70.3
5	-	n.d.	4‘‘	3.16 (m)	72.2
6	6.43 (s)	99.8	5‘‘	3.63 (m)	70.3
7	-	n.d.	6‘‘	0.93 (d, 5.8)	18.0
8	6.72 (s)	95.0			
9	-	n.d.	*Ara*		
10	-	n.d.	1‘‘‘	4.16 (d, 5.9)	106.2
1‘	-	n.d.	2‘‘‘	3.36 (m)	71.1
2‘	7.37 (s)	115.9	3‘‘‘	3.32 (m)	72.6
3‘	-	164.0	4‘‘‘	3.58 (br s)	67.8
4‘	-	150.4	5‘‘‘	3.51 (m), 3.29 (m)	65.8
5‘	6.89 (d, 7.7)	116.2			
6‘	7.29 (d, 7.7)	121.4	*Glc*		
			1‘‘‘‘	5.06 (d, 6.9)	100.6
			2‘‘‘‘	3.28 (m)	73.3
			3‘‘‘‘	3.33 (m)	76.5
			4‘‘‘‘	3.20 (m)	69.8
			5‘‘‘‘	3.44 (m)	77.3
			6‘‘‘‘	3.72 (d, 11.5), 3.48 (m)	60.8

[a)] Rha = *α*-L-rhamnopyranosyl; Ara = *α* -L-arabinopyranosyl; Glc = *β*-D-glucopyranosyl
[b)] ^{13}C NMR shifts derived from HSQC and HMBC data
[c)] n.d. not detected

Table 2 ^{1}H and ^{13}C NMR data of compound **4** in DMSO-d_6

position	δ_H (m, *J* in Hz)	δ_C [b),c)]	position[a)]	δ_H (m, *J* in Hz)	δ_C [b)]
Aglycone			*Rha*		
2	-	157.4	1‘‘	5.23 (br s)	101.5
3	-	135.0	2‘‘	4.06 (br s)	80.8
4	-	n.d.	3‘‘	3.66 (dd, 9.3, 3.2)	70.8
5	-	162.0	4‘‘	3.15 (dd, 9.5, 9.4)	72.4
6	6.22 (s)	97.6	5‘‘	3.81 (dq, 10.0, 6.4)	70.7
7	-	164.8	6‘‘	0.95 (d, 6.4)	17.8
8	6.42 (s)	92.5			
9	-	156.8	*Ara*		
10	-	104.3	1‘‘‘	4.09 (d, 6.7)	106.9
1‘	-	119.9	2‘‘‘	3.31 (dd, 8.9, 6.7)	71.6
2‘	6.93 (s)	107.9	3‘‘‘	3.29 (dd, 9.0, 2.9)	73.0
3‘	-	146.5	4‘‘‘	3.55 (br s)	68.1
4‘	-	137.9	5‘‘‘	3.44 (dd, 12.0, 2.0), 3.23 (br d, 12.0)	66.2
5‘	-	146.5			
6‘	6.93 (s)	107.9			

[a)] Rha = *α*-L-rhamnopyranosyl, Ara = *α* -L-arabinopyranosyl
[b)] ^{13}C NMR shifts derived from HSQC and HMBC data
[c)] n.d. not detected

Legends for Figures

Fig. 1 HPLC-PDA-ESIMS of the MeOH extract of *B. pinnatum*. Top: UV trace (220-400 nm). Bottom: ESIMS base peak chromatogram (positive ion mode, *m/z* 200 - 1500). SunFire^{TM} C_{18} column, A: 0.1% aqueous formic acid and B: MeCN, 5-100% B in A in 30 min, 0.5 mL/min.
Numbers refer to the isolated compounds **1-11.** Letters refer to α-linolenoyl lysophosphatidylcholine (**a**, tentative assignment), linoleoyl lysophosphatidylcholine and an isomer (**b, c**), and palmitoyl lysophosphatidylcholine (**d**), respectively. Peaks at R_t 19.5 and 20.0 min (both *m/z* 699.7), 22.6 min (*m/z* 537.6) and 22.7 (*m/z* 677.7) could not be identified. The front peak in the UV trace contains large amount of malic acid, as revealed by ^{1}H NMR analysis.

Fig. 2 Structures of compounds isolated from *B. pinnatum.*

Fig. 3 Structures of bufadienolides isolated from *B. daigremontianum* and detected in *B. pinnatum*.

Fig. 4 Presence of bufadienolides in the CH_2Cl_2 soluble fraction of *B. pinnatum*: HPLC-APCIMS base peak chromatogram (positive ion mode, *m/z* 150 - 1500). SunFire^{TM} C_{18} column, A: 0.1% aqueous formic acid and B: MeCN, 5-100% B in A in 30 min, 0.5 mL/min. Numbers refer to bufadienolides **12-15.** * This peak (*m/z* 475.3) was tentatively assigned to bryophyllin C [34]. Peaks at R_t 11.0 min (*m/z* 197.2) and R_t 11.3 min (*m/z* 477.3, 459.3) were not identified.

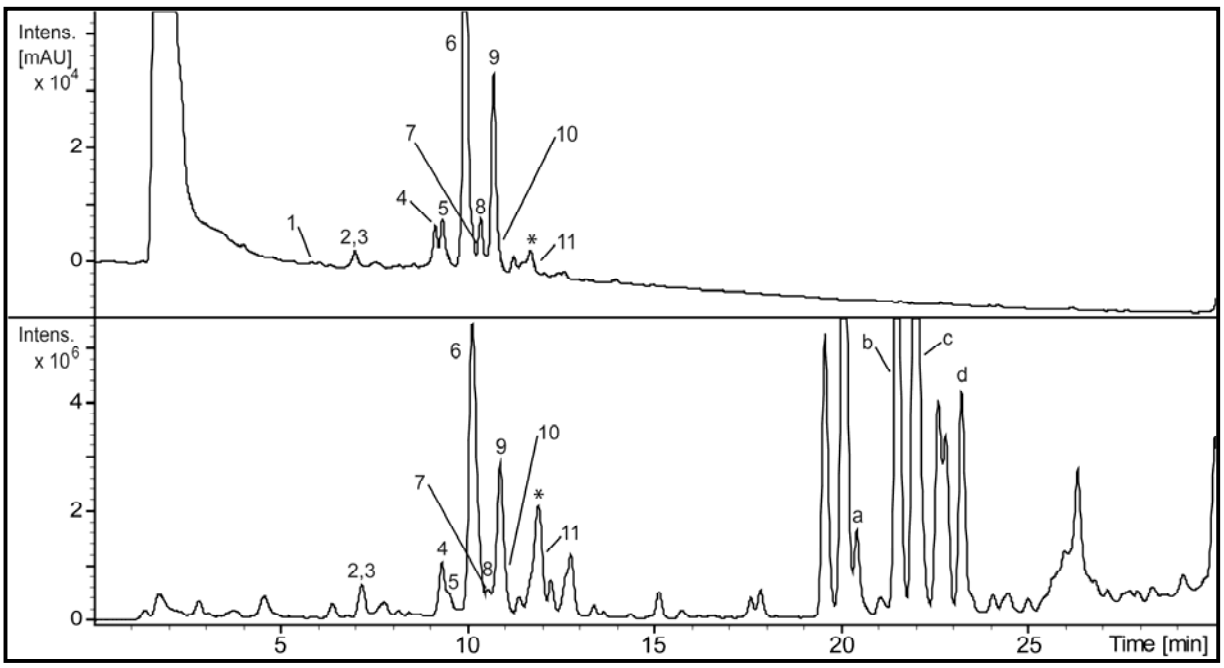


Fig. 1 HPLC-PDA-ESIMS of the MeOH extract of *B. pinnatum*. Top: UV trace (220-400 nm). Bottom: ESIMS base peak chromatogram (positive ion mode, *m/z* 200 - 1500). SunFire^{TM} C_{18} column, A: 0.1% aqueous formic acid and B: MeCN, 5-100% B in A in 30 min, 0.5 mL/min.

Numbers refer to the isolated compounds **1-11**. Letters refer to α-linolenoyl lysophosphatidylcholine (**a**, tentative assignment), linoleoyl lysophosphatidylcholine and an isomer (**b, c**), and palmitoyl lysophosphatidylcholine (**d**), respectively. Peaks at R_t 19.5 and 20.0 min (both *m/z* 699.7), 22.6 min (*m/z* 537.6) and 22.7 (*m/z* 677.7) could not be identified. * This peak contains an unidentified flavonoid with *m/z* 507. The front peak in the UV trace contains large amount of malic acid, as revealed by ^{1}H NMR analysis.

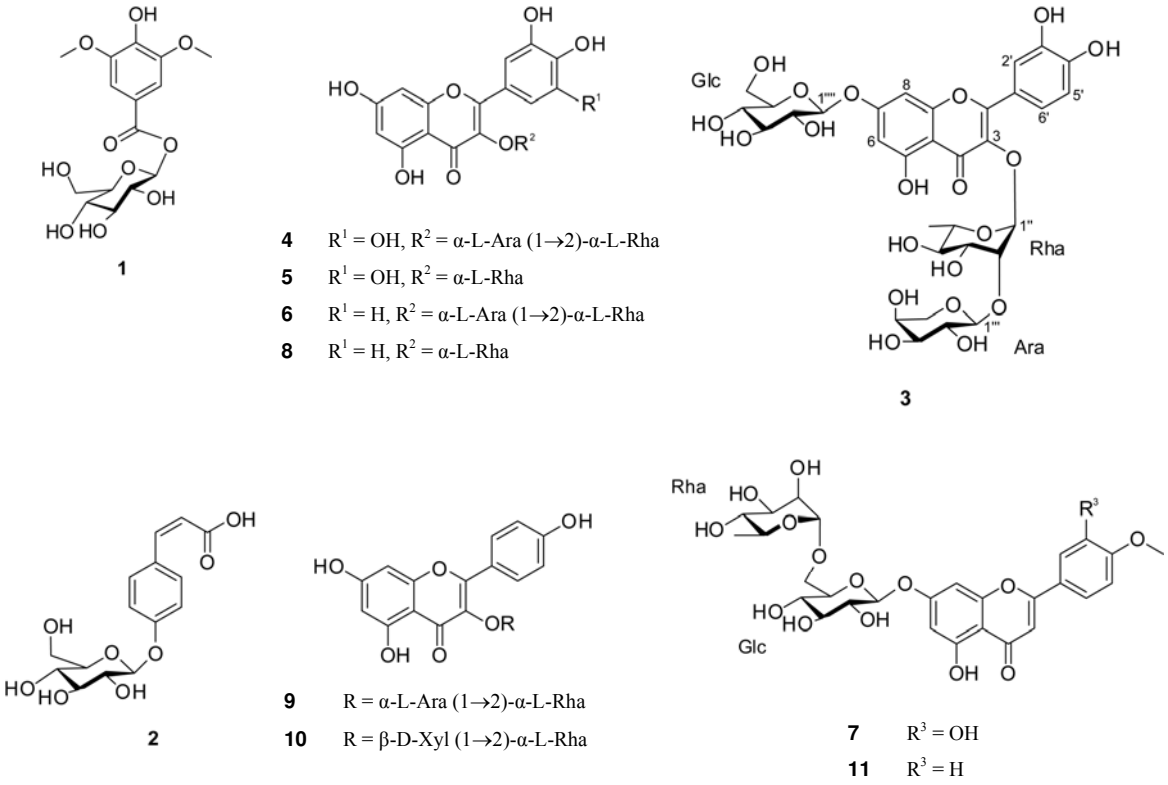
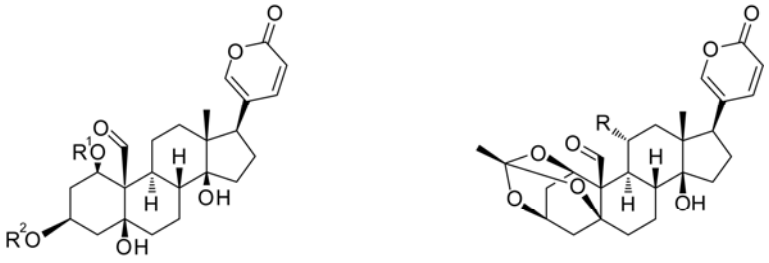


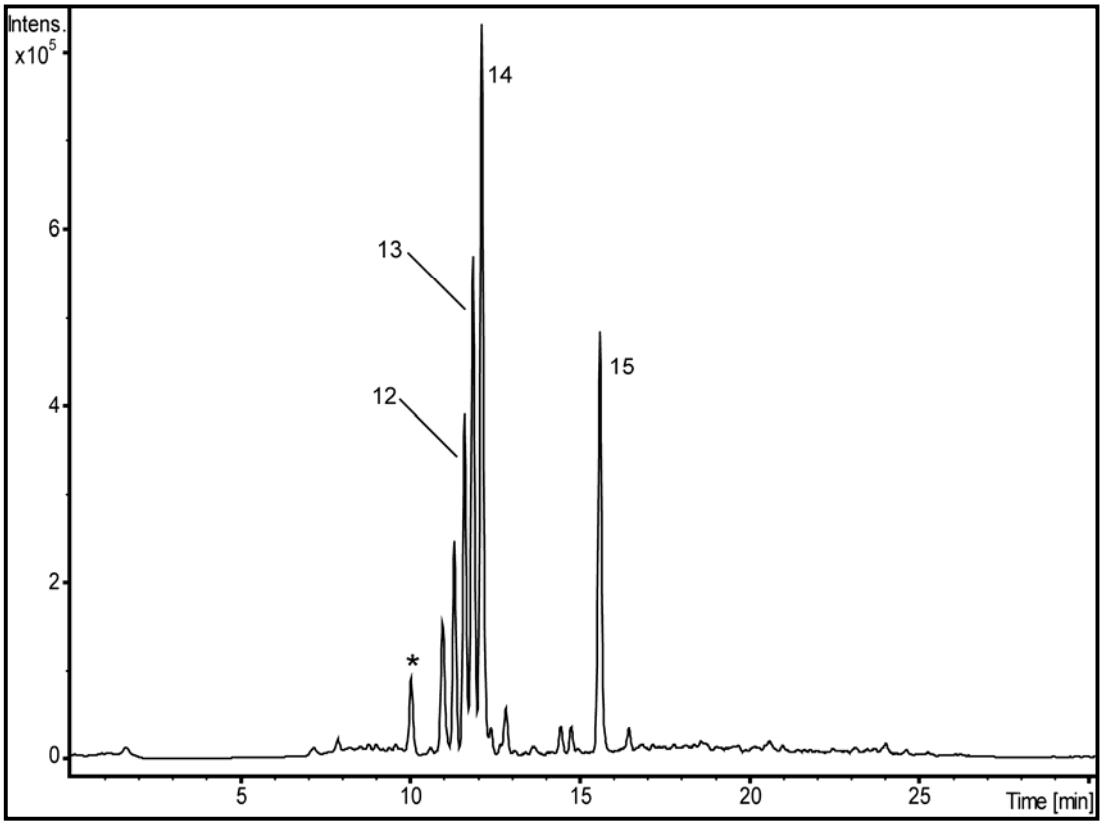
Fig. 2 Structures of compounds isolated from *B. pinnatum*



12 R^1 = Ac, R^2 = H
14 R^1 = H, R^2 = Ac

13 R = OH
15 R = H

Fig. 3 Structures of bufadienolides isolated from *B. daigremontianum* and detected in *B. pinnatum*.



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