Bryophyllum pinnatum - metabolite profiling and *in vitro* effects on porcine detrusor contractility

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“Wie aus einem Blatt unzählig frische Lebenszweige spriessen, mögst in einer Liebe selig tausendfaches Glück geniessen!”

Goethe, 1830

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Abbreviations

AC	Adenylyl cyclase
ACh	Acetylcholine
AM	Anthroposophical medicine
APCI	Atmospheric-pressure chemical ionisation
AP1	Activator protein-1
ASA	Acetylsalicylic acid
AUC	Area under the curve
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
DAG	Diacylglycerine
DO	Detrusor overactivity
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
EBV-EA	Epstein-Barr virus early antigen
EFS	Electrical field stimulation
EMSA	Electrophoretic mobility shift assay
ER	Extended release
ESI	Electrospray ionisation
EvaMed	Evaluation of Anthroposophic Medicine
GABA	γ-Aminobutyric acid
GOT	Glutamyl pyruvate transaminase
GPT	Glutamyl oxaloacetic acid transaminase
HPLC	High-performance liquid chromatography
HPV	Human papillomavirus
HRQL	Health-related quality of life
ICIQ-OAB	International Consultation on Incontinence Modular Questionnaire for OAB
ICS	International Continence Society
i.v.	Intravenous
i.p.	Intraperitoneal
IP_3	Inositol-1,4,5-triphosphate
IR	Immediate release
KHQ	King's Health Questionnaire
LDA	Limiting dilution analysis
LUT	Lower urinary tract
MIC	Minimum inhibitory concentration
MLC	Myosin light chain

MLCK	Myosin light chain kinase
MT	Mother tincture
MUI	Mixed urinary incontinence
NANC	Non-adrenergic non-cholinergic
NO	Nitric oxide
OAB	Overactive bladder
OVA	Ovalbumin
PDA	Photodiode array
PDE5	Phosphodiesterase type 5
PG	Prostaglandin
PKA	Protein kinase A
PLC	Phospholipase C
PMC	Pontine micturition centre
p.o.	per os
PPi	Pyrophosphate
PUSC	Pontine urine storage centre
ROCK	Rho-kinase
QoL	Quality of life
SALP	Serum alkaline phosphatase
SBLN	Serum bilirubin
SGOT	Serum glutamyl oxaloacetic acid transaminase
SGPT	Serum glutamyl pyruvate transaminase
SR	Sarcoplasmic reticulum
UTI	Urinary tract infection

Summary

Bryophyllum pinnatum is a succulent perennial plant that belongs to the family of Crassulaceae and is originally from Madagascar. *B. pinnatum* is widespread in tropical areas worldwide and has been used in traditional medicine. In Europe, Rudolf Steiner introduced *B. pinnatum* in anthroposophical medicine for the first time in 1921. Nowadays, *B. pinnatum* is a phytotherapeutic that is available in the form of leaf press juice and leaf extracts. Besides other potential indications, *B. pinnatum* is used and examined for the treatment of hyperactive conditions. Since 1970, *B. pinnatum* has been used successfully as a tocolytic agent in various hospitals. The efficacy could be confirmed by several empirical as well as by clinical studies. *In vitro* experiments demonstrated a relaxant effect of the aqueous leaf extract, leaf press juice, and the flavonoid fraction of *B. pinnatum* on spontanous human myometrial contractions. An additional experiment also showed a relaxant effect of the aqueous leaf extract on oxytocin-induced contractions.

Based on the fact that *B. pinnatum* has a relaxant effect on uterine smooth muscle, the inhibitory effect on urinary bladder smooth muscle was investigated. Overactive bladder (OAB) is a symptomatic syndrome, which affects many individuals and is defined as urinary urgency, with or without urge incontinence, usually with frequency and nocturia. Antimuscarinic drugs are the first-line pharmacotherapy prescribed to OAB patients. Due to anticholinergic side effects and insufficient therapeutic effects, an alternative phytopharmaceutical treatment is increasingly desired. Recently, a randomised, double-blind placebo-controlled study demonstrated a positive impact of *B. pinnatum* 50% chewable tablets against placebo by reducing the micturition frequency over 24 hours. In initial experiments, the *B. pinnatum* leaf press juice (5 - 10%) demonstrated a maximum relaxant effect on carbachol pre-contracted detrusor of 18.7% and an inhibition of electrically induced contractility by 74.6%.

For the administration of a phytotherapeutic, it is essential to have extensive knowledge about the constituents and their therapeutic effects and toxicity. This thesis describes the isolation and identification of *B. pinnatum* constituents in a methanolic leaf extract. Two phenolic acid derivatives and nine known flavonoid glycosides were isolated, including two new natural products belonging to the flavonoids, namely quercetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside 7-O-*β*-D-glucopyranoside and myricetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside. Bufadienolides, a substance group with sedative, positive inotropic and CNS-related activities is known in other *Bryophyllum* species, e.g. *B. daigremontianum*, and has been detected in the methanolic extract of *B. pinnatum* only in trace amounts. For this reason, four reference substances were isolated

from *B. daigremontianum* and subsequently, bersaldegenin-1-acetate, bryophyllin A, bersaldegenin-3-acetate, and bersaldegenin-1,3,5-orthoacetate were detected in *B. pinnatum*.

Based on these findings, the methanolic leaf extract of *B. pinnatum* has been fractionated in a flavonoid, bufadienolide, and polar fraction. The effect of these three fractions on electrically stimulated porcine detrusor contractions was evaluated and compared with oxybutynin. The flavonoid fraction demonstrated a dose- and time-dependent inhibition of detrusor muscle contractility and a significant maximum inhibition of 78.7% using a 1 mg/mL concentration. The inhibitory effect of the flavonoid fraction was comparable with the maximum inhibition produced by the reference substance oxybutynin (10^{-6} M) at 78.1%. Bladder strips treated with the bufadienolide fraction (0.1 - 40 µg/mL) did not produce any inhibitory effect on detrusor contractility. The polar fraction demonstrated an unexpected dose-dependent inhibition. However, the observed effect may be explained by decreased pH caused by L-malic acid present in this fraction. These results suggest that flavonoids are important for the inhibition of detrusor contractility.

To examine the effect of *B. pinnatum* on human detrusor muscles, human bladder wall samples were obtained from patients after radical cystectomy. The *in vitro* experimental results confirmed the observed inhibitory effect of the leaf press juice and the flavonoid fraction; the effect seems to be more potent as the inhibition is observed at lower concentrations than with porcine detrusor strips.

The insights gained from the intensive inhibition of porcine and human detrusor contractility by *Bryophyllum pinnatum* are promising and support further investigations for the treatment of hyperactive conditions.

Zusammenfassung

Bryophyllum pinnatum ist eine mehrjährige sukkulente Pflanze die zur Familie der Dickblattgewächse (Crassulaceae) gehört und ursprünglich aus Madagaskar stammt. Heutzutage ist B*. pinnatum* in nahezu allen tropischen Gebieten der Welt zu finden und wird dort in der traditionellen Medizin eingesetzt. In Europa wurde *B. pinnatum* im Jahre 1921 durch Rudolf Steiner in die anthroposophische Medizin eingeführt. Heute ist *B. pinnatum* in Form des Blattpresssaftes oder der Blattauszüge ein Phytotherapeutikum, das neben anderen potentiellen Indikationen zur Behandlung von hyperaktiven Zuständen eingesetzt wird bzw. untersucht ist. Seit 1970 wird *B. pinnatum* in verschiedenen Kliniken erfolgreich als Tokolytikum bei vorzeitiger Wehentätigkeit verabreicht. Die Wirksamkeit konnte durch mehrere empirische aber auch klinische Studien bestätigt werden. In *in vitro* Experimenten zeigten der wässrige Blattextrakt, der Blattpresssaft, sowie eine Flavonoid-Fraktion aus *B. pinnatum* Blättern eine Relaxation der spontanen Kontraktionen des humanen Myometriums. In einem weiteren Experiment konnte eine relaxierende Wirkung des wässrigen Blattextraktes auf Oxytocin-induzierte Kontraktionen gezeigt werden.

Basierend auf der Tatsache, dass *B. pinnatum* eine relaxierende Wirkung auf die glatte Muskulatur des Uterus hat, wurde der hemmende Effekt auf den glatten Muskel der Harnblase untersucht. Die hyperaktive Blase (Reizblase) ist in der Bevölkerung weit verbreitet und definiert sich als imperativer Harndrang mit oder ohne Dranginkontinenz und tritt häufig mit Pollakisurie und Nykturie auf. Als Ersttherapie werden den Patienten antimuskarinische Medikamente verschrieben; aufgrund anticholinerge Nebenwirkungen sowie ungenügender Wirkung sind alternative Therapiemöglichkeiten z.B. im Bereich der Phytotherapie zunehmend gefragt. Kürzlich zeigte eine randomisierte, placebokontrollierte doppelblinde Studie einen positiven Trend von *B. pinnatum* 50% Kautabletten gegenüber Placebo in Form einer Abnahme der Miktionsfrequenz über 24 Stunden. Erste Experimente mit *B. pinnatum* Presssaft (5 - 10%) am Schweinedetrusormuskel *in vitro* haben eine maximale relaxierende Wirkung auf den Carbachol-vorstimulierten Detrusormuskel von 18.7% und eine Hemmung der Kontraktilität des elektrisch stimulierten Muskels um 74.6% gezeigt.

Für den Einsatz von Phytotherapeutika ist ein umfassendes Wissen über die Inhaltstoffe der Pflanze sowie deren Wirkung und Toxizität essentiell. In der vorliegenden Arbeit wird die Isolierung und Identifizierung von Substanzen aus dem methanolischen Blattextrakt von *B. pinnatum* beschrieben. Es wurden zwei Phenolsäurederivate und verschiedene bereits bekannte Flavonoidglykoside sowie zwei neue Naturstoffe aus der Gruppe der Flavonoide, nämlich Quercetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranosid 7-O-*β*-D-

glucopyranosid und Myricetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranosid isoliert. Bufadienolide, eine Substanzgruppe mit sedativer sowie positiv inotroper Wirkung und bekannt v.a. aus anderen *Bryophyllum* Arten wie z.B. *B. daigremontianum,* wurden im *B. pinnatum* Methanolextrakt nur in geringsten Mengen detektiert. Aus diesem Grund wurden aus *B. daigremontianum* Referenzsubstanzen isoliert; damit konnten in *B. pinnatum* letztlich Bersaldegenin-1-acetat, Bryophyllin A, Bersaldegenin-3-acetat und Bersaldegenin-1,3,5-orthoacetat detektiert werden.

Basierend auf diesem Wissen wurde der methanolische Blattextrakt von *B. pinnatum* in eine Flavonoid-, Bufadienolid- und polare Fraktion aufgetrennt. Der Effekt dieser drei Fraktionen wurde auf die Kontraktilität des elektrisch stimulierten Detrusormuskels gegenüber Oxybutynin getestet. Die Flavonoid-Fraktion zeigte eine dosis- und zeitabhängige Hemmung der Detrusorkontraktilität; bei einer Konzentration von 1 mg/ml wurde die maximale Hemmung von 78.7% erreicht. Der hemmende Effekt der Flavonoid-Fraktion auf die Muskelkontraktilität war vergleichbar mit der maximalen Hemmung der Referenzsubstanz Oxybutynin (10^{-6} M) von 78.1%. Die Bufadienolid-Fraktion (0.1 - 40 µg/ml) zeigte keine hemmende Wirkung auf die Kontraktilität der Muskelstreifen. Hingegen zeigte unerwartet auch die polare Fraktion eine dosis-abhängige Hemmung, die jedoch durch die enthaltene L-Apfelsäure und den damit verbundenen pH-Abfall im Organbad erklärt werden konnte. Diese Resultate deuten darauf hin, dass die Flavonoide für die Hemmung der Detrusorkontraktilität wichtig sind.

Um den Effekt von *B. pinnatum* auch auf den humanen Detrusormuskel zu testen, wurden bei Patienten nach radikaler Zystektomie Blasenwandproben entnommen. Die bisherigen Resultate bestätigen den hemmenden Effekt des Presssaftes und der Flavonoid-Fraktion im Schweinemuskelmodell; die Wirkung scheint jedoch potenter zu sein, da die Effekte bei geringeren *B. pinnatum* Konzentrationen auftreten als beim Schweinemuskelstreifen.

Die gewonnenen Erkenntnisse über die intensive Hemmung der Kontraktilität des humanen und des Schweinedetrusormuskels durch *B. pinnatum* sind äusserst vielversprechend und unterstützen weitere Untersuchungen zur Anwendung von *B. pinnatum* bei hyperaktiven Zuständen.

1 AIM OF THE WORK

The phytotherapeutic *Bryophyllum pinnatum* is approved by the Swiss Agency for Therapeutic Products, Swissmedic, as a medicine without any indication. Physicians prescribe *B. pinnatum* preparations for a wide spectrum of diagnosis and some therapeutic effects of *B. pinnatum* are confirmed by empirical and clinical studies: However, little is known about the mechanism of action.

In a first step, we had to gain detailed knowledge about the phytochemical composition of *B. pinnatum*: Constituents had to been isolated from a methanolic leaf extract using appropriate chromatographic procedures. Furthermore, the structures of isolated compounds had to be elucidated by HPLC-PDA-MS analyses and NMR spectroscopy.
The previously observed relaxant effect of the leaf press juice on myometrial smooth muscle was the reason why further investigations were focused on the detrusor muscle. Overactive bladder syndrome is a hyperactive disorder and affects many individuals. Nowadays, antimuscarinic drugs are used as first-line pharmacotherapy. However, the treatment often goes with undesirable anticholinergic side effects; Hence, looking for alternative treatments is ongoing; the phytotherapeutic *B. pinnatum* could be one possibility. In a second part of this thesis, the effect of the leaf press juice and its fractions on porcine and human detrusor contractility shall been investigated *in vitro*.

2 GENERAL INTRODUCTION

2.1 *Bryophyllum pinnatum*

2.1.1 Botanical systematics

Bryophyllum pinnatum (Lamarck) Oken (syn. *Kalanchoe pinnata* (Lamarck) Persoon), synonym: *Bryophyllum calycinum* (Salisbury) belongs to the family of Crassulaceae and is known by numerous vernacular names, such as life plant, air plant, love plant, miracle leaf, cathedral bells, and Goethe plant. *B. pinnatum* is a succulent perennial plant that is native to Madagascar and also grows in different tropical areas of Africa, America, Indonesia, India, China, and Australia.

“Alles in Einem und aus Einem” glaubt ich mit Augen zu sehen.

Goethe, 1826

Figure 1. *B. pinnatum* plantlet [1]

This herbaceous plant has a rectangular, hollow stem and reaches a height of 1 - 1.5 m. The decussate arranged leaves are succulent and fleshy dark green (Fig. 2). The lower leaves are scalloped and trimmed in red. The upper leaves are 3 - 7 foliate. The ovate-oblong leaves are approximately 10 - 20 cm long and have notches bearing a dormant bud. Rooting vegetative buds on the leaf margin are visible and able to develop into a healthy plantlet when the leaf falls to the ground (Fig. 1).

Figure 2. *B. pinnatum* plant [1]

In contrast to the sexual reproduction of most flowering plants, *B. pinnatum* has a rather unique mode of vegetative reproduction, whereby young plantlets develop on the edges of leaves before being shed for propagation. Despite the enormous vegetative force that is needed for reproduction, the plant develops gorgeous inflorescences from November to March, and fruits in April [2].

The pendulous flowers are coloured violet on top then fade from green to reddish and consist of a tubular and inflated calyx (2 - 4 cm). The ciliated corolla is longer than the calyx, which is coloured purple with eight basally located stamens arranged in two circles (Fig. 3). There are four carpels (6 - 12 mm), connate only at the base,

with styles 2 - 3.5 cm long. The flower builds four follicles containing numerous ovoid seeds, which are barely germinable. After flowering, new side shoots replace the flowers [2,4,5].

Figure 3. *B. pinnatum* blooming [3]

B. pinnatum is uniquely adapted to arid conditions, as it employs the typical metabolism of CAM-plants (Crassulacean Acid Metabolism). The stomata in the leaves remain closed during the day to protect the plant from water loss. At night, stomatal opening allows CO_2 uptake and storage as malate in vacuoles. During the day, malate is released from the vacuoles and decarboxylated to free CO_2. Entering the Calvin cycle, CO_2 is bound to ribulose-1,5-bisphosphate and converted into carbohydrates. This process enables photosynthesis to occur when stomata are closed [6,7].

2.1.2 History

In 1783, Jean-Baptiste Lamarck (1744-1829) described *Cotylet pinné* in the "Encyclopédie méthodique" for the first time. He had previously reported the wound-healing, pain-relieving, and refreshing effects of this plant.

After the first specimen had been imported from India (Calcutta) to England, Christian Hendrik Persoon (1755-1837) named the plant *Calanchoe pinnata* and Richard Anthony Salisbury (1762-1829) described the plant as *Bryophyllum calycinum*. During the following Continental Blockade (1806-1814) the exchange of knowledge was stopped and scientists described the plant independently.

Based on the "Botanical Magazine; or Flower-Garden Displayed" from William Curtis, Johann Wolfgang von Goethe (1749-1832) became interested in *B. calycinum* and recorded his observations in his diary for the first time in 1818. He began to investigate *B. calycinum* extensively, including its metamorphosis and its first blossoming [5,8]. Years after Goethe's death, his manuscripts and studies were summarised and published by Georg Balzer in his book "Goethe's *Bryophyllum*. Ein Beitrag zu seiner Pflanzenmorphologie" (Fig. 4) [10].

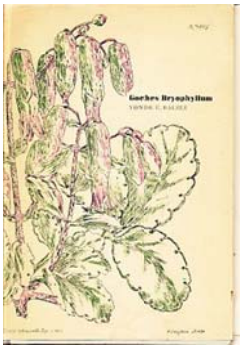


Figure 4. Goethe's *Bryophyllum* [9]

In addition to *B. pinnatum* another related species, *Bryophyllum daigremontianum*, was introduced to Europe circa 1900 [5].

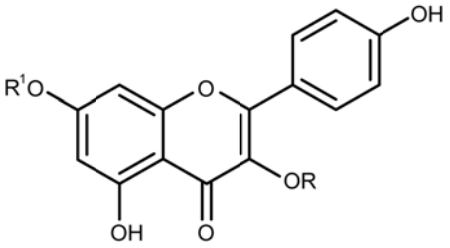
2.1.3 Phytochemical constituents

The known phytochemicals present in *B. pinnatum* include flavonoids, alkaloids, various phenolics, triterpenoids, steroids, bufadienolides, lipids, fatty acids, minerals, and vitamins [2]. Various compounds have been identified in *B. pinnatum*, specifically from its leaves.

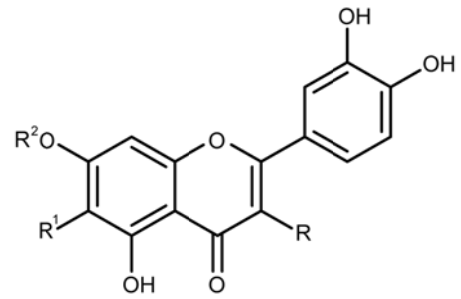
Flavonoids

Flavonoids have demonstrated broad therapeutic effects for various indications. A number of flavonoids, mainly quercetin and kaempferol glycosides, have been previously identified. Several studies have revealed the presence of the following flavonoids in *B. pinnatum* (Fig. 5):

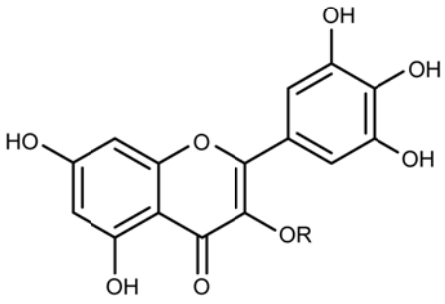
Kaempferol (**1**) [2], kapinnatoside (kaempferol 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside (**2**) [11], kaempferol 3-O-*β*-D-xylopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside (**3**) [12], kaempferitrin (**4**), kaempferol 3-O-*α*-L-(2-acetyl)rhamnopyranoside 7-O-*α*-L-rhamnopyranoside (**5**), kaempferol 3-O-*α*-L-(3-acetyl)rhamnopyranoside 7-O-*α*-L-rhamnopyranoside (**6**), kaempferol 3-O-*α*-L-(4-acetyl)rhamnopyranoside 7-O-*α*-L-rhamnopyranoside (**7**), kaempferol 3-O-*α*-D-glucopyranoside 7-O-*α*-L-rhamnopyranoside (**8**), afzelin (**9**), *α*-rhamnoisorobin (**10**) [13], kaempferol 3-*β*-D-glucopyranoside (**11**) [14], astragalin (**12**), quercetin (**13**) [2], quercetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside (**14**), quercitrin (**15**), quercetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside 7-O-*β*-D-glucopyranoside (**16**) [12], quercetin 3-diarabinoside [14], rutin (**17**) [15], luteolin (**18**) [16], luteolin 7-O-*β*-D-glucoside (**19**) [15], myricitrin (**20**), myricetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside (**21**), diosmine (diosmetin 7-*O*-*α*-L-rhamnopyranosyl-(1 → 6)-*β*-D-glucopyranoside, **22**), acacetin 7-O-*α*-L-rhamnopyranosyl-(1 → 6)-*β*-D-glucopyranoside (**23**) [12], 4',5-dihydroxy-3',8-dimethoxyflavone 7-O-*β*-D-glucopyranoside (**24**) [11], 3',4'-dimethoxy quercetin (**25**) [17], 3,5,7,3',5'-pentahydroxyflavone (structural inconsistencies) [18], 3,8-dimethoxy-4',5,7-trihydroxyflavone (**26**) [2], epigallocatechin-3-O-syringate (**27**) [16], as well as two anthocyanidins 5'-methyl-4',5,7-trihydroxyl flavone (**28**) and 4',3,5,7-tetrahydroxy-5-methyl-5'-propenamine anthocyanidine (**29**) [19] shown in figure 6.



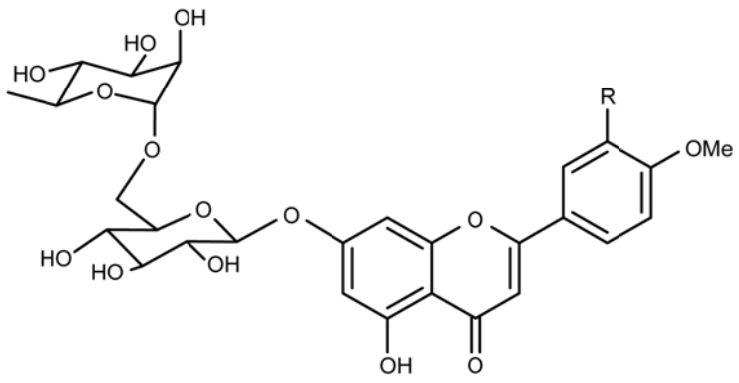
	R	R^1
1	H	H
2	α-L-Ara-(1 → 2)-α-L-Rha	H
3	β-D-Xyl-(1 → 2)-α-L-Rha	H
4	α-L-Rha	α-L-Rha
5	α-L-Rha-^{2}OAc	α-L-Rha
6	α-L-Rha-^{3}OAc	α-L-Rha
7	α-L-Rha-^{4}OAc	α-L-Rha
8	β-D-Glc	α-L-Rha
9	α-L-Rha	H
10	H	α-L-Rha
11	H	β-D-Glc
12	β-D-Glc	H



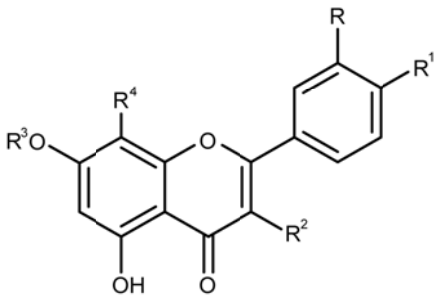
	R	R^1	R^2
13	OH	H	H
14	O-α-L-Ara-(1 → 2)-α-L-Rha	H	H
15	O-α-L-Rha	H	H
16	O-α-L-Ara-(1 → 2)-α-L-Rha	H	β-D-Glc
17	O-α-L-Rha-(1 → 6)-β-D-Glc	H	H
18	H	H	H
19	H	H	β-D-Glc



	R
20	α-L-Rha
21	α-L-Ara-(1 → 2)-α-L-Rha



	R
22	OH
23	H



	R	R^1	R^2	R^3	R^4
24	OMe	OH	OH	β-D-Glc	OMe
25	OMe	OMe	OH	H	H
26	H	OH	OMe	H	OMe

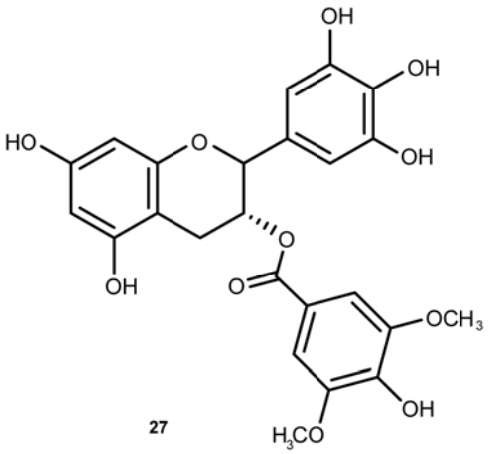


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Figure 5. Flavonoids identified in *B. pinnatum*

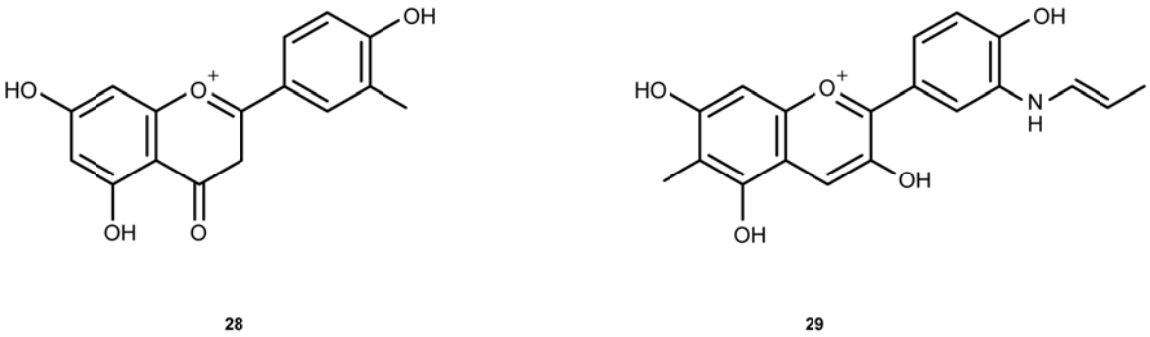


Figure 6. Anthocyanidins identified in *B. pinnatum*

Anthocyanidines belong to the flavonoid family and are the aglyca of anthocyanins. These plant pigments are responsible for the red, purple and blue colours of most flowers, as well as the colour of fruits, leaves, stems, and roots [20].

In a recent study, the distribution of anthocyanins was observed in seedlings of *B. pinnatum* under full, 70, 50, and 25% sunlight. A higher peripheral accumulation of anthocyanins in the stems and petioles of *B. pinnatum* under full sunlight was observed compared with 25% light (Fig. 7). The ability of anthocyanins to protect plants from UV damage, also known as the photoprotective effect, has been confirmed [21].

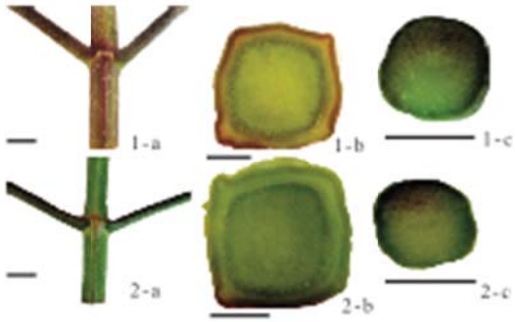


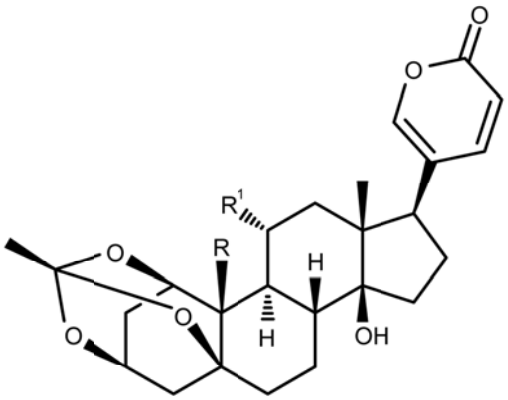
Figure 7. Distribution of anthocyanins in sections of stems (a, b) and petiole (c) of *B. pinnatum* seedlings under full sunlight (1a, b, c) and 25% light (2a, b, c). Scale bars are shown for a) 10 mm and for b, c) 5 mm [21].

Bufadienolides

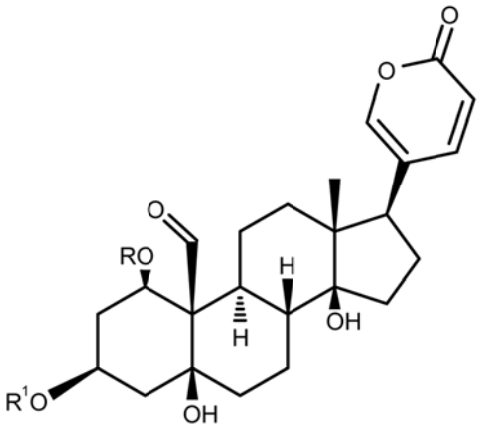
Bufadienolides possess structures and activities similar to cardiac glycosides. Bufadienolides in *Bryophyllum* species are known to have sedative, positive inotropic properties, as well as CNS-related, antitumor, and insecticidal activities. The pattern of bufadienolides in *Bryophyllum* has species-dependent qualitative and quantitative differences [22-25]. Previous phytochemical investigations revealed the presence of the following bufadienolides in *B. pinnatum* (Fig. 8): Bryophyllin A (bryotoxin C, **30**), bryophyllin B (**31**) [23], bryophyllin C (**32**) [24], bersaldegenin-1-acetate (**33**) [12], bersaldegenin-3-acetate (**34**) [23], bersaldegenin-1,3,5-orthoacetate (**35**) [26], bryotoxin A (**36**), and bryotoxin B (**37**) [25].

In other species, additional bufadienolides have been identified. Daigredorigenin [27], daigremontianin, and daigredorigenin-3-acetate were isolated from *B. daigremontianum* [22]

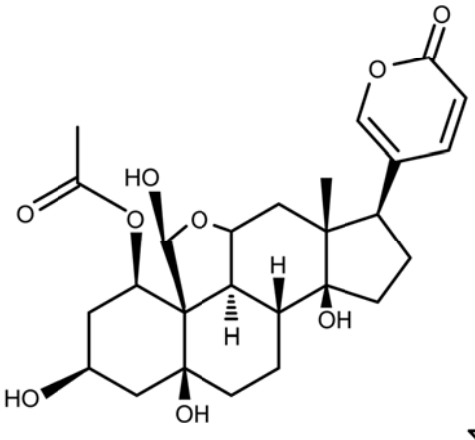
and kalantuboside A and B from *B. tubiflorum* [28]. In their hybrid, *B. daigremontianum* x *D. tubiflorum,* another bufadienolide, methyl daigremonate, was identified [29]. Further, kalanchoside A, B, C, thesiuside, hellebrigenin, and hellebrigenin-3-acetate were found in *Kalanchoe gracilis* [30] and kalanhybrin A, B, C, and daigredorigenin-3-acetate in *K. hybrida* [29].



	R	R^1
30	CHO	OH
32	CH_2OH	OH
35	CHO	H



	R	R^1
33	Ac	H
34	H	Ac



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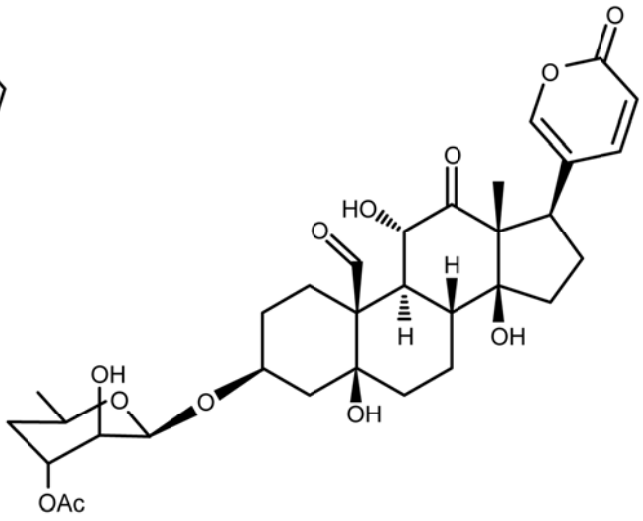

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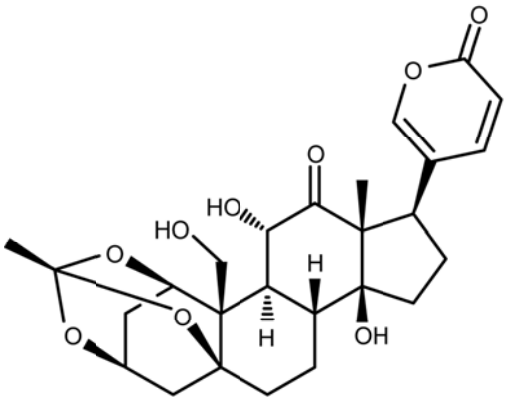

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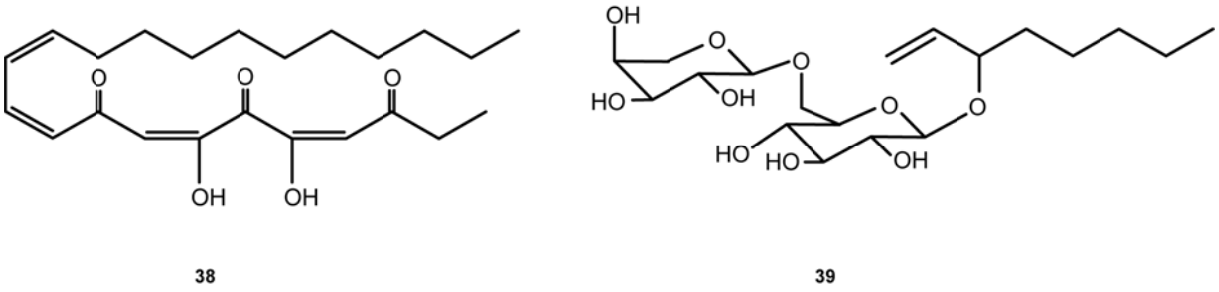
Figure 8. Bufadienolides identified in *B. pinnatum*

Alkanes, alkanols, triterpenes and steroids

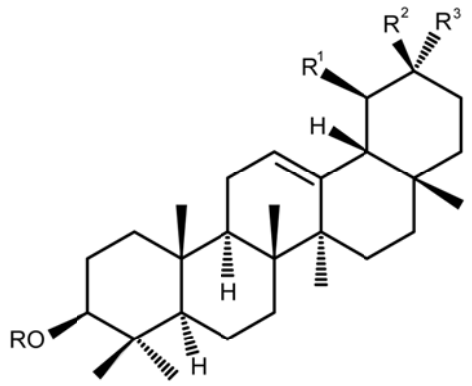
The presence of *n*-alkanes and *n*-alkanols, such as bryophollenone (**38**) [31], and of a minor constituent, 1-octen-3-O-*α*-L-arabinopyranosyl-(1 → 6)-glucopyranoside (**39**), has also been demonstrated in *B. pinnatum* [32,33] (Fig. 9).



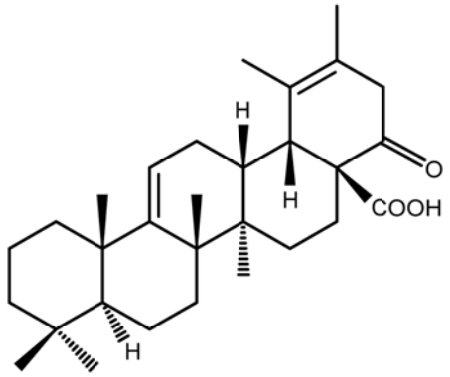
38 39

Figure 9. Alkanes identified in *B. pinnatum*

Furthermore, several triterpenes, such as *α*-amyrin (**40**), *β*-amyrin (**41**) [32], *α*-amyrin-*β*-D-glucopyranoside (**42**) [34], bryophollone (**43**), bryophynol (**44**), 18*α*-oleanane (**45**), *Ψ*-taraxasterol (**46**) [31], taraxerol (**47**) [2], taraxerone (**48**), glut-5(6)-en-3-one, 3*β*-friedelanol (**49**) [18], friedelin (**50**), glutinol (**51**) [2] were detected in *B. pinnatum* (Fig. 10, 11).



	R	R^1	R^2	R^3
40	H	CH_3	H	CH_3
41	H	H	CH_3	CH_3
42	β-D-Glc	H	CH_3	CH_3



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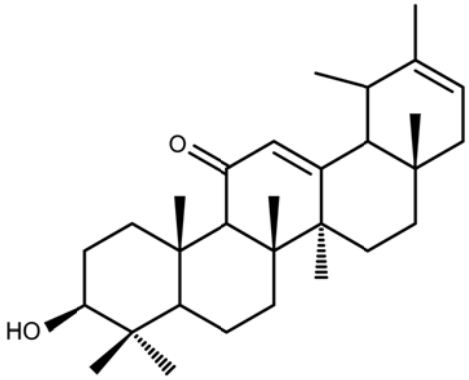

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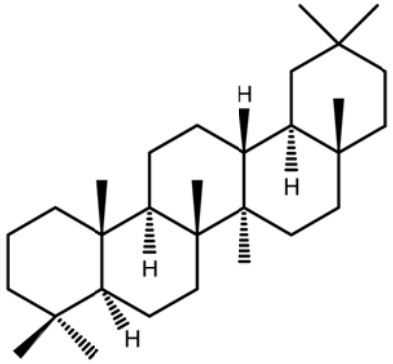

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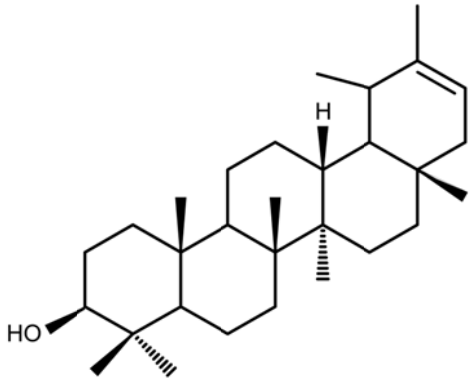

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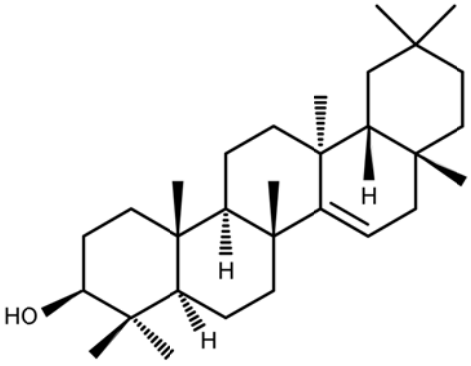

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Figure 10. Triterpenes identified in *B. pinnatum*

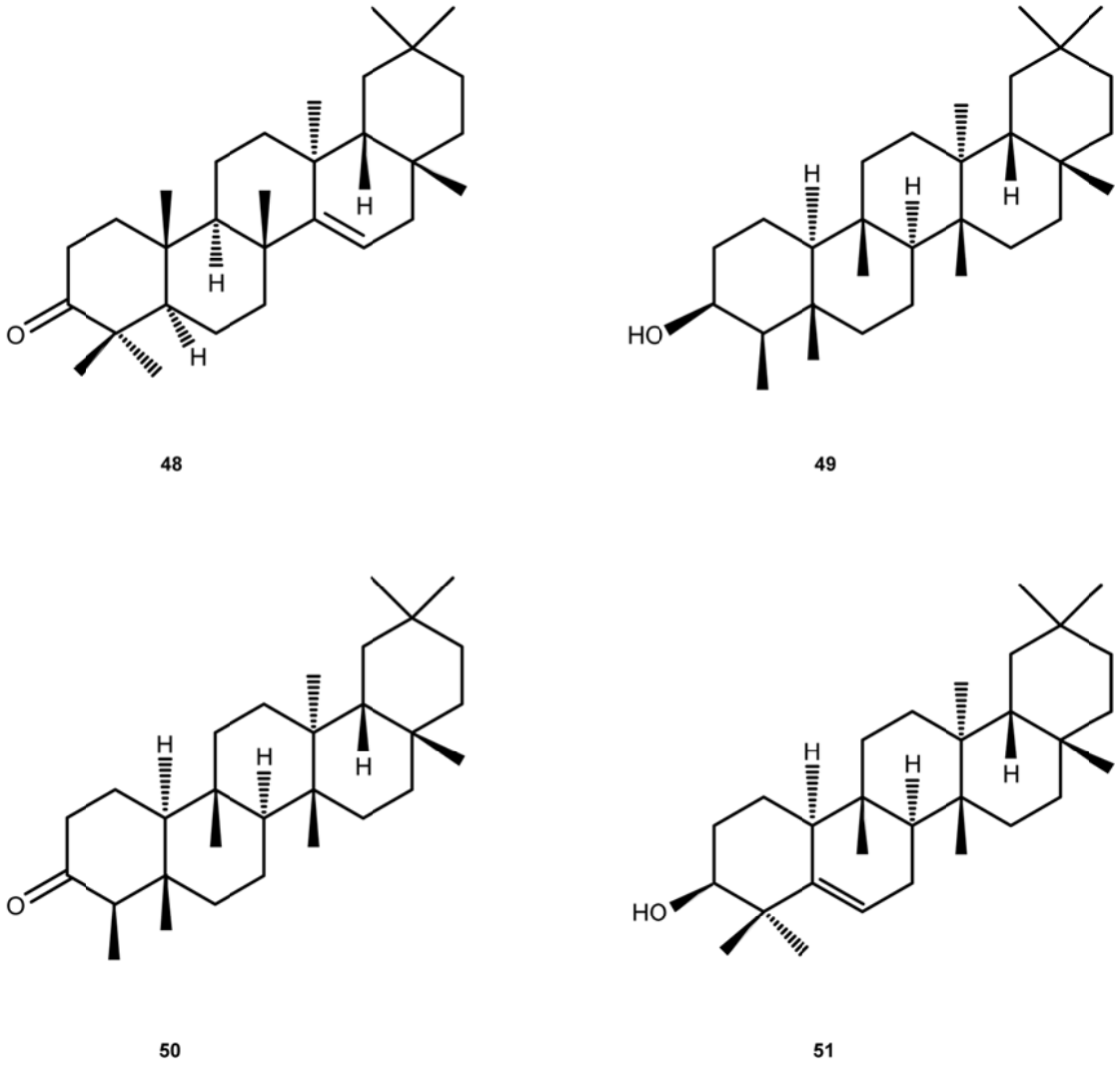

Figure 11. Triterpenes identified in *B. pinnatum*

Phytosterols, such as bryophyllol (**52**) [31], stigmast-24-enol (**53**), (24S)-stigmast-25-enol (**54**), 25-methylergost-24(28)-enol (**55**), clerosterol (**56**), 24-epiclerosterol (**57**), *β*-sitosterol (**58**), 22-dihydrobrassicasterol (**59**), stigmasterol (**60**), campesterol (**61**), isofucosterol (**62**), codisterol (**63**), 24-ethyl-desmosterol (**64**), ergosta-5,24(28)-dienol (**65**), 25-methylergosta-5,24(28)-dienol (**66**) [35], 24-ethyl-25-hydroxycholesterol (**67**) [31], clionasterol (**68**) [2], peposterol (**69**), avenasterol (**70**), (24R)-stigmasta-7,25-dienol (**71**), (24S)-stigmasta-7,25-dienol (**72**) [35], stigmast-4,20(21),23-trien-3-one (**73**) [34] were identified in *B. pinnatum* (Fig. 12-14).

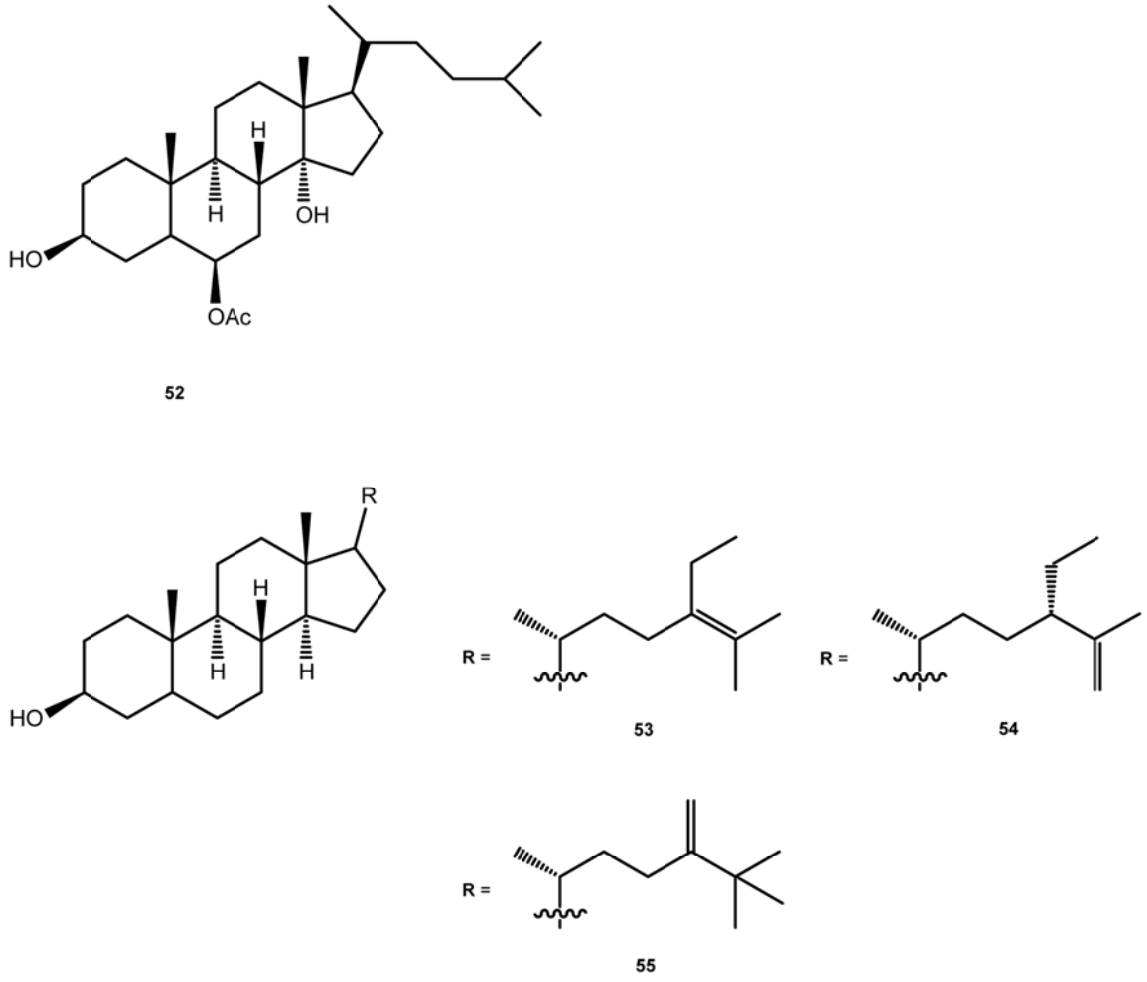

Figure 12. Phytosterols identified in *B. pinnatum*

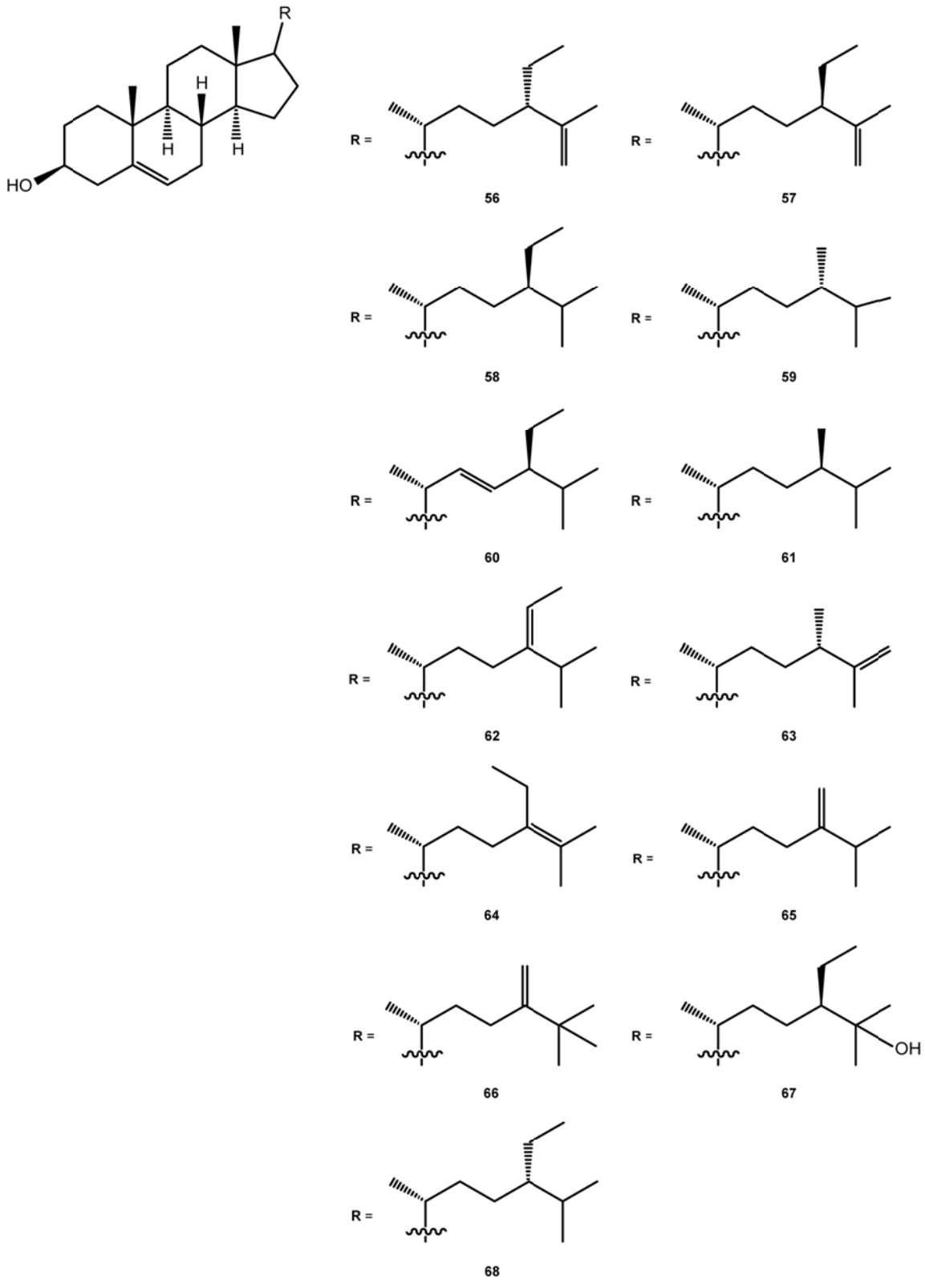

Figure 13. Phytosterols identified in *B. pinnatum*

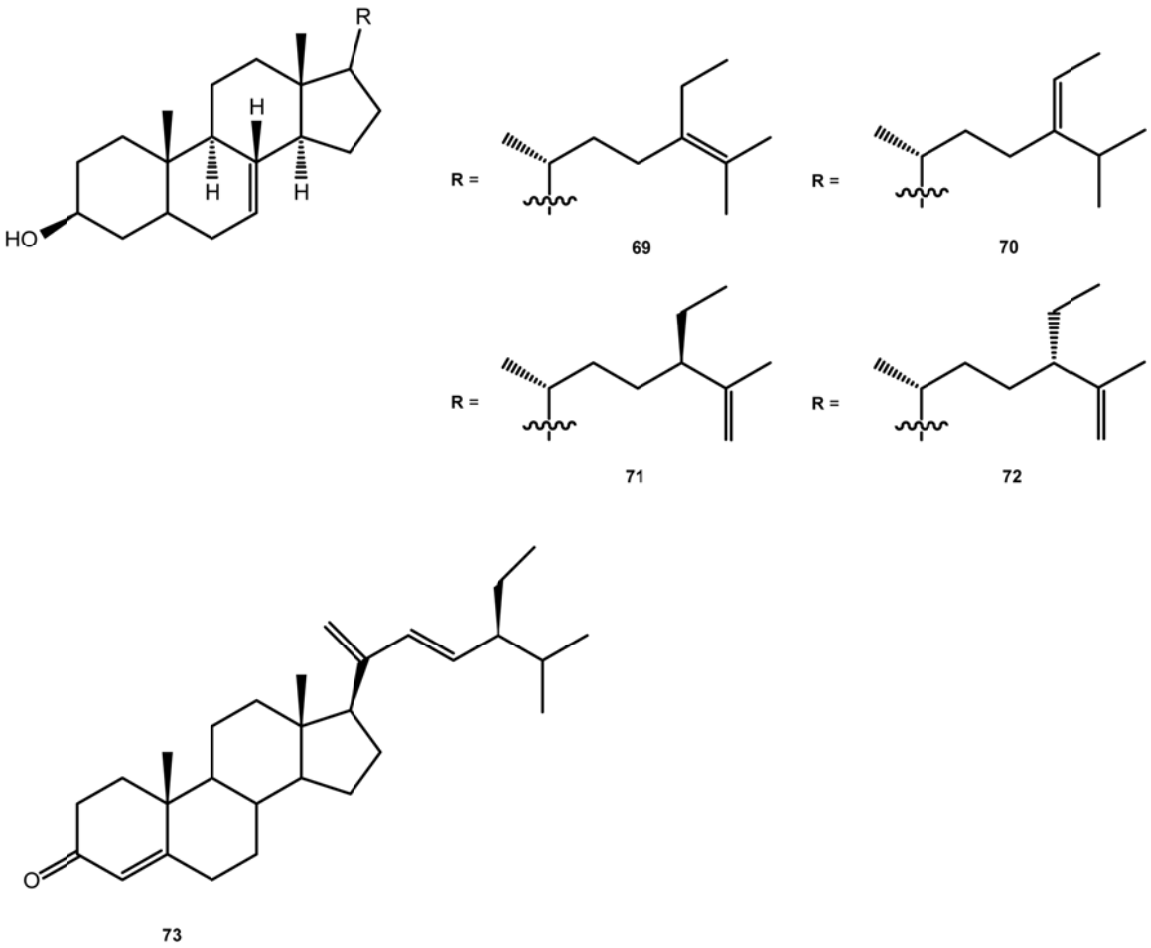

Figure 14. Phytosterols identified in *B. pinnatum*

Phenanthrenes

2(9-decenyl)-phenanthrene (**74**), 2-(9-undecenyl)-phenanthrene (**75**) [31], 1-ethanamino-7-hex-1-yne-5'-one phenanthrene (**76**) [36] were identified in *B. pinnatum* (Fig. 15).

Phenolic and carboxylic acids

Several phenolic acid derivatives are constituents of *B. pinnatum*: gallic acid [16], ferulic acid, syringic acid, caffeic acid, *p*-coumaric acid, protocatechuic acid, 4-hydroxy-3-methoxy-cinnamic acid [2], syringic acid *β*-D-glucopyranosyl ester (**77**), 4'-O-*β*-D-glucopyranosyl-*cis*-*p*-coumaric acid (**78**) [2], and *p*-hydroxybenzoic [37]. Additionally, various carboxylic acids are contained in *B. pinnatum,* such as malic acid, oxalic acid, citric acid, isocitric acid, cinnamic acid, succinic acid, oxaloacetate, and phosphoenolpyruvate (Fig. 15) [2].

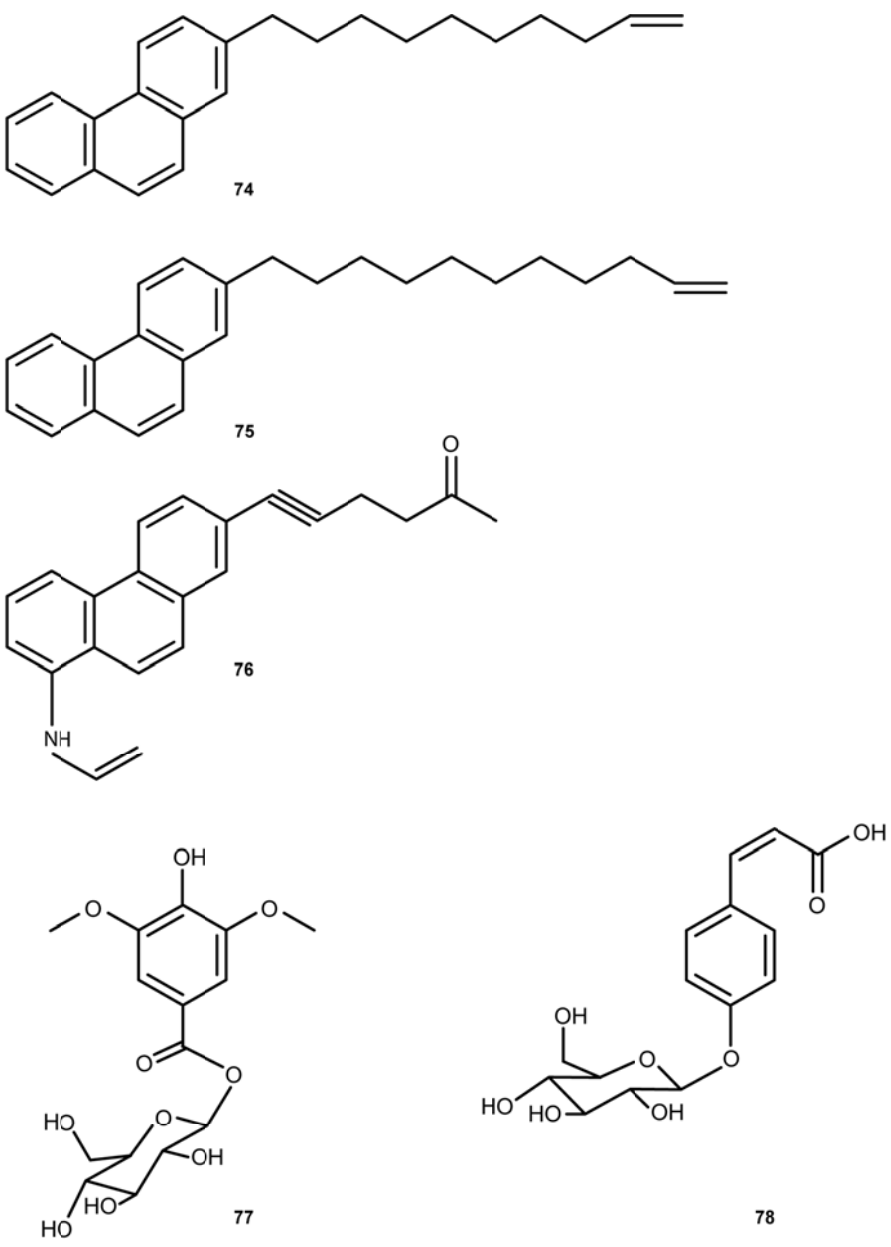

Figure 15. Other constituents identified in *B. pinnatum*

Fatty acids, minerals and other constituents

The following fatty acids, minerals, vitamins, and other substances have also been identified in *B. pinnatum*: palmitic acid, stearic acid, arachidic, behenic acid [38], *n*-hentriacontane, *n*-tritriacontane [37], HCN, vitamins (ascorbic acid, riboflavin, thiamine, niacin, pyridoxine), amino acids (cysteine, glutamic acid, glycine, methionine, phenylalanine, tyrosine) casein hydrolysate, protein hydrolysate, carbohydrates, protein, lipids, acids, minerals (sodium, calcium, iodine, potassium, phosphor, magnesium, manganese, iron, copper, zinc), and different sugars [2].

2.1.4 Ethnopharmacology

B. pinnatum has been widely used in traditional medicine, particularly in hot and moist regions of the world where the plants grow spontaneously, such as Madagascar, Nigeria, Trinidad and Tobago, India, Indonesia, Philippines, Indo-China, and Brazil. The leaves and stems are bitter and, due to their astringent effects, they are effective against diarrhoea, flatulence, and vomiting [2]. Currently, the leaves are used to treat several diseases and afflictions, such as hypertension, kidney and urinary disorders [39], cough, or to prevent asthma symptoms [40]. In India, the leaves are used as a hepatoprotective medicine to treat jaundice [41]. Furthermore, the leaf extracts of *B. pinnatum* have antiulcer [42], antihistamine [43], antimicrobial [44], antibacterial [45], anti-inflammatory, antinociceptive, hypoglycaemic [46], and antipyretic activity, as well as antimalarial effects [47].

2.1.5 Anthroposophical medicine

Anthroposophical medicine (AM) is an integrative multimodal medical system that was developed by the Austrian philosopher Rudolf Steiner (1861-1925) and the Dutch medical doctor Ita Wegman (1876-1943). AM is currently practiced in 80 countries worldwide.

In 1921, the first anthroposophical hospital in Arlesheim, Switzerland, was established by Dr Ita Wegman. AM is practiced by physicians that are fully trained and qualified in university medicine integrating conventional skills and methods with a holistic understanding of man and nature. From this point of view, the understanding of the human being in his entirety means the acceptance of a three system organisation with a physical body, the soul and the spirit. This holistic view of the human being leads to an understanding of health and illness that differs from conventional medicine and to treatments that are specifically adapted to each individual.

How does AM describe and interpret *B. pinnatum* and its character?

B. pinnatum has an exceptional ability to let grow new plants from its leaves. Due to this process, we can consider that one leaf contains the whole plant and that it has a strong vegetative force, with the tendency toward reproduction that occurs from out of the leaves demonstrating great vitality. Additionally, flower formation is somehow displaced to the leaves, as demonstrated by the distribution of anthocyanins, which are normally present in flowers, and are responsible for the reddish and purple patterns on the leaves. Furthermore, the plant only begins to flower under favourable conditions.

From an AM perspective, *B. pinnatum* is therapeutically indicated if the so-called astral and etheric bodies separate too much from each other. That means that the processes linked to psychic qualities, such as emotions (represented by the astral body), and physiological

processes (represented by the etheric body) that are basis for the health of a patient are not balanced.

B. pinnatum reunites these two parts of the human organisation to restore holistic balance. Based on this principle, *B. pinnatum* has historically been used to treat inner restlessness and anxiety and, therefore, was also called “herbal valium” due to its sedative properties [5,8].

In 1921, *B. pinnatum* preparations were initially recommended by Rudolf Steiner as anthroposophical medicines to treat hysteria [48]. He described hysteria as the condition if the whole spiritual and emotional energy is spent into an action and therefore the body is not capable anymore to regulate normal physical reactions [49]. In 1970, Dr Werner Hassauer introduced *B. pinnatum* as a tocolytic agent to prevent premature labour in AM hospitals. From an AM viewpoint, an imbalance in the astral and etheric organisations can lead to cramps, vaginal infections and, therefore, to premature labour. The healthy interaction of astral and etheric bodies is supported by *B. pinnatum*. Dr Hassauer demonstrated that treatment with *B. pinnatum* could decrease the dosage of betamimetics, or even replace them, and was also well tolerated in women with premature labour [50].

Investigations concerning the effect of *B. pinnatum* on several hyperactive conditions are described in Chapter 1.1.7.1.

2.1.6 *B. pinnatum* preparations and application

Currently, various *B. pinnatum* preparations are commercially available. A large multi-centre observational study was performed by 38 German physicians collaborating in the Evaluation of Anthroposophic Medicine (EvaMed) network. Over 6 years, a total of 4038 prescriptions were recorded in the EvaMed data bank and showed a broad indication range [51]. *B. pinnatum* preparations are mainly produced by Weleda AG, while WALA Heilmittel GmbH focuses on the taxonomically related *B. daigremontianum*.

The application of *B. pinnatum* preparations is described in the German Commission C monographs. In Switzerland, *B. pinnatum* preparations are authorised by the Swiss Agency for therapeutic products (Swissmedic) as a medicinal product without any indication.

B. pinnatum is used for the treatment of premature labour and for other medical conditions, such as sleep disorders induced by restlessness, anxiety, pain induced by vital weakness, and recurrent inflammation in the metabolic system [52]. Combined preparations, such as *B. pinnatum* Mercurio cultum or Argento cultum contain plants were fertilized with the corresponding homeopathic diluted metal (quicksilver or silver). These preparations are primarily used to regulate metabolic processes with or without concomitant psychological symptoms (e.g., restlessness, sleep disorders, constraints). The combination of *B. pinnatum* and Conchae is also used to harmonise rhythm and are prescribed to patients suffering from

difficulty falling asleep, restlessness, excitation and exhaustion [53]. *B. pinnatum* preparations from Weleda AG are available in different galenical forms. The usual dosage of preparations in Switzerland and Germany are shown in Table 1.

Table 1. *Bryophyllum pinnatum* preparations from Weleda AG. Usual dosages are shown [52-55].

Monopreparation	Dosage form	Usual dosage	Weleda
Bryophyllum 50%	Chewable tablets	Tocolysis acute (1st day): 1 tablet each 15 min for 1h, continue with 1 tablet each 2h Tocolysis (after 1 day): 1-2 tablets 3-4 times per day Daily sedation (pregnancy): 1 tablet 3-4 times per day, 2 tablets before sleeping Sleep problems: 2 tablets before sleeping, 1 tablet as a reserve	CH
Bryophyllum 5%	Ampoules 1 mL	Injections (i.m., s.c.) twice a week until once a day	CH, D
Bryophyllum Ø (= 33%)	Dilutio	Adults: 15-20 drops 1-3 times per day Children: 5-10 drops 1-3 times per day	CH, D
Bryophyllum D1	Dilutio	15-20 drops 1-3 times per day	CH, D
Bryophyllum D5	Dilutio	15-20 drops 1-3 times per day	CH
Bryophyllum Argento cultum 1% (in D: D2)	Dilutio	Adults and young people aged under 12 years: 10-15 drops 2-4 times per day Children from 6 to 11 years: 8-10 drops 1-3 times per day Small children from 2-5 years: 5-8 drops 1-3 times per day	CH, D
Bryophyllum Argento cultum 0,1% (in D: D3)	Dilutio		
Bryophyllum Argento cultum Rh D2	Ampoules 1 mL	Injections (s.c.) twice a week until once a day	CH, D
Bryophyllum Argento cultum Rh D3	Ampoules 1 mL		
Bryophyllum Argento cultum Rh D3	Dilutio aquosa	Adults and young people aged under 12 years: 10-15 drops 2-4 times per day Children from 6 to 11 years: 8-10 drops 1-3 times per day Small children from 2-5 years: 5-8 drops 1-3 times per day	CH, D
Bryophyllum Mercurio cultum 1% (in D: D2)	Dilutio	5-10 drops 1-3 times a day	CH, D
Bryophyllum Mercurio cultum 0,1% (in D: D3)	Dilutio		
Bryophyllum Rh D3	Ampoules 1 mL	Injections (s.c.) twice a week	D
Bryophyllum Rh D3	Dilutio aquosa	5-10 drops 1-3 times a day	D
Bryophyllum Mercurio cultum Rh D3	Ampoules 1 mL	Injections (s.c.) twice a week until once a day	D
Bryophyllum Mercurio cultum Rh D3	Dilutio aquosa	5-10 drops 1-3 times a day	D
Bryophyllum 50%	Pulvis	2 knife points of powder 3 times per day Babies: 1 knife point of powder dissolved in tea 3 times per day	D

Combination preparation	Dosage form	Usual dosage	
Bryophyllum D5 / Conchae D7 aa	Ampoules 1 mL	Injections (s.c.) 2-3 times a week until once a day (evenings)	CH, D
Bryophyllum D5 / Conchae D7 aa 10ml	Ampoules 10 mL	Acute situation: Injection (i.v.)	CH, D
Bryophyllum D5 / Conchae D7 aa	Dilutio	10-20 drops 1-3 times per day	CH
Bryophyllum 50% / Conchae 5% aa	Pulvis	1 knife point (max. 1/2 teaspoon) 1-3 times per day	CH
Bryophyllum 50% / Conchae 50% aa	Pulvis	1 knife point 1-3 times per day	CH
Bryophyllum 50% 9T / Conchae 50% 1T	Pulvis	1 knife point (max. 1/2 teaspoon) 1-3 times per day	CH
Argentum D6 / Bryophyllum 50% aa	Pulvis	1 knife point 1-3 times per day	CH
Cimicifuga comp.	Dilutio	10-20 drops 1-3 times per day before eating	CH, D

CH = Arlesheim, Switzerland; D = Schwäbisch Gmünd, Germany

2.1.7 Pharmacological activities

B. pinnatum is a well-regarded plant with a high phytotherapeutic potential. The leaves are particularly promising for the treatment of various disorders.

2.1.7.1 Hyperactivity disorders

As described previously, *B. pinnatum* has been used to treat various disorders, based on hyperactive conditions. *In vitro* and *in vivo* research activities that have been performed in this field are listed in the following section (detailed information are provided in Table 2).

Tocolysis

B. pinnatum preparations have been used as a tocolytic agent since years and several studies have been performed.

Dr Hassauer showed that the tocolysis with *B. pinnatum* 5% infusion and 50% trituratio was well tolerated and successful in 84% of the women. The treatment could also decrease the dosage of the beta-agonist fenoterol, or even replace them [50].

A retrospective study including 170 pregnant women investigated the tocolytic effect of *B. pinnatum*. Group A was treated with *B. pinnatum* 5% infusion followed by the oral treatment with trituratio 50%. The treatment in group B started also with *B. pinnatum* 5% infusion and due to an inadequate effect after 2 hours, women were additionally treated with fenoterol intravenous (i.v.) or per os (p.o.) followed by the treatment with trituratio 50%. *B. pinnatum* showed a comparable positive outcome to fenoterol and no side effects were registered [56].

From 1977 until 2000, a total of 1622 deliveries were documented and evaluated by Dr Istvan Vilàghy. The study revealed 253 pregnant women, who needed a tocolytic therapy as well as 29 premature deliveries (1.8%) were registered. In the period from 1977 to 1983, fenoterol was used to prevent premature labour resulting in an incidence of premature deliveries of 6.2%. In the following years, he integrated *B. pinnatum* in the treatment of premature labour. From 1990 to 2000, Dr Vilàghy treated pregnant women almost exclusively with *B. pinnatum* 33% dilutio. Since *B. pinnatum* was used for the treatment of premature labour, the incidence of premature deliveries decreased to 1.07% [57].

In a retrospective study, the tolerability and tocolytic activity of i.v. administered *B. pinnatum* 5% compared with beta-agonists (fenoterol or hexoprenaline) were investigated in 67 pregnant women. This study demonstrated similar maternal and neonatal outcomes in both treatment groups. However, maternal adverse effects (palpitation, dyspnoea) were significantly reduced and the use of corticosteroids and antibiotics was lower in the group treated with *B. pinnatum*. The neonatal outcomes and morbidity rates were similar or

superior in the *B. pinnatum* treatment group [58]. In addition, a prospective, randomised clinical trail assessed the efficacy and safety of *B. pinnatum* 50% chewable tablets versus a currently used calcium antagonist, nifedipine, for the treatment of premature contractions. A total of 27 pregnant women were included and showed no change in bishop score from visit 1 (0 - 1h) to visit 2 (4h). The treatment with *B. pinnatum* as well as with nifedipine revealed a significant decrease in the number of contractions/h of 5.1 and 4.5, respectively. The neonatal outcome did not differ between the two groups. However, a significant shortening of hospital stay was observed with *B. pinnatum* [59].

Furthermore, the tocolytic activity of *B. pinnatum* observed in anthroposophical medicine was confirmed in an *in vitro* study compared with fenoterol. *B. pinnatum* aqueous leaf extract (10^4 mg/L) demonstrated a dose-dependent inhibition of spontaneous human myometrium contractions, although the contraction frequency increased. The extract also demonstrated a relaxant effect on oxytocin-induced contractions. Fenoterol decreased myometrial contractions and frequency [60]. Additionally, the effects on spontaneous contractions in myometrial strips by *B. pinnatum* leaf press juice, and tentatively identified flavonoid, bufadienolide, and cinnamic acid fractions was investigated *in vitro*. After five regular muscle contractions, 2 µL of the leaf press juice (undiluted) and the fractions (1%, 2%, 5%, 10%, undiluted) was added to the organ bath chamber. The effect on the area under the curve (AUC), amplitude, and frequency of the contractions was measured. The leaf press juice significantly reduced the AUC to 82% and rapidly increased the frequency to 128% after the first application. The reduction of the amplitude to 78% was statistically significant after the second application. The flavonoid fraction (undiluted) significantly reduced the AUC to 51% and caused a rapid and large increase in the frequency to 557% after the first application. The amplitude was reduced to 70% after the second application. The two other fractions did not significantly affect the AUC and the amplitude, but increased the contraction frequency more than the control [61].

The mechanism of the tocolytic effect of *B. pinnatum* was further investigated using human myometrial cells. Firstly, the leaf press juice demonstrated a dose-dependent inhibition of the oxytocin-induced increase of $[Ca^{2+}]_i$ in hTERT-C3 cells (IC_{50} = 0.94%) and also a somewhat weak inhibitory effect in M11 cells. Furthermore, in hTERT-C3 cells, in extracellular Ca^{2+}-free conditions, the press juice was able to significantly inhibit the oxytocin-induced increase of $[Ca^{2+}]_i$. Therefore, the tocolytic effect is assumed to be independent of extracellular calcium concentration. Secondly, the effect of the leaf press juice was investigated using SH-SY5Y human neuroblastoma cells, which express voltage-dependent L-type Ca^{2+} channels. The $[Ca^{2+}]_i$ response to KCl was not reduced by the press juice, however, the voltage-dependent Ca^{2+}-influx through L-type Ca^{2+} channels was delayed [62].

Overactive bladder

In a prospective, randomised, double-blind placebo-controlled study, 20 postmenopausal women suffering from overactive bladder (OAB) or urgency-dominant mixed urinary incontinence (MUI) were treated with *B. pinnatum* 50% chewable tablets or placebo. The women took 3 x 2 blinded capsules during 8 weeks. In a total of 15 weeks, they had five visits and were ask to fill out 2-day bladder diaries and answer two questionnaires, the King's Health Questionnaire (KHQ) and the International Consultation on Incontinence Modular Questionnaire for OAB (ICIQ-OAB). After treatment, a positive trend for *B. pinnatum* was observed compared to placebo. The primary endpoint, the micturition frequency/24h, was reduced from 9.5 before to 7.8 after the treatment (p=0.064). The quality of life (QoL) was comparable in the *B. pinnatum* and placebo group [63].

The effect of *B. pinnatum* leaf press juice on porcine detrusor muscle contractility was investigated in an organ bath chamber versus oxybutynin. The press juice (5% in the chamber) significantly inhibited detrusor contractility induced by electrical field stimulation (EFS) by 74.6% compared to the control. In addition, the press juice (10%) demonstrated a significant relaxant effect (18.7%) on carbachol-induced contractions. The leaf press juice demonstrated a good activity, although oxybutynin showed a greater inhibition and relaxation of the detrusor muscle (detailed information are provided in Chapter 4.1) [64].

Additional *in vitro* experiments have investigated the effect of leaf press juice and different fractions of *B. pinnatum* on electrically induced porcine detrusor contractility. The inhibitory effect of the leaf press juice was successfully confirmed. The flavonoid fraction reduced muscle contractility in a dose- and time-dependent manner and produced a significant maximum inhibition of 78.7% using a 1 mg/mL concentration. The bufadienolide fraction had no inhibitory effect on contractility at the investigated concentrations and the polar fraction showed an unexpected inhibitory effect; however, this effect may be explained by decreased pH in the organ bath chamber due to the presence L-malic acid (detailed information are provided in Chapter 4.2) [65].

Sleep disorders

In a prospective, multi-centre, observational study, 49 pregnant women suffering from sleep disorders were treated with *B. pinnatum* 50% chewable tablets. The women took 3 - 8 tablets per day and were asked to complete questionnaires before and after the 14-day treatment. The number of wake-ups and the subjective quality of sleep was significantly improved and they were less sleepy during the day. However, a prolongation of sleep duration and reduction in the time to fall asleep was not achieved [66].

Neurological disorders

The behavioural neuropharmacology of the *B. pinnatum* aqueous leaf extract was investigated in mice. The results demonstrated neurosedative, CNS depressant, and anxiolytic activities. Furthermore, a dose-dependent muscle relaxant effect of the aqueous extract was observed, which was comparable to diazepam. Diazepam is a benzodiazepine that increases the effect of γ-aminobutyric acid (GABA) by binding to the $GABA_A$ receptor and finally results in a sedative, anticonvulsant, anxiolytic and muscle relaxant effect. Therefore, it is suggested, that *B. pinnatum* may have GABAergic activity [67]. Some of these neuropharmacological effects were tested in Swiss mice applying a methanolic fraction. Specifically, the GABA content in the brain was estimated after i.p. administration. It was demonstrated that the methanolic fraction induces an increase in brain GABA concentration [68].

In an early study, the neurosedative effect of bersaldegenin-1,3,5-orthoacetate was demonstrated in mice. A strongly sedative activity was observed with doses of 0.1-0.5 mg/mL. The CNS was affected after higher doses and resulted in paralysis and muscle contractions [27]

Table 2. Investigations of the effect of *B. pinnatum* on hyperactivity disorders.

Hyperactivity disorder	**Preparation/ Substance**	**Study design/ Bioassay**		**Effect**	
Tocolysis	*Bryophyllum* 5% infusion *Bryophyllum* 50% trituratio	*in vivo*	Retrospective study: *B. pinnatum* vs. fenoterol Group A: *B. pinnatum* (n=19; 2 women got only the oral therapy) Group B: *B. pinnatum* + fenoterol (n=31) Dose: infusion 5% (until cessation of contractions) + trituratio 3 x 1 knife point p.o. followed by 1 knife point each 1-2h Group B, alternative if therapy is ineffective: fenoterol infusion followed by tablets (or only tablets)	Group A: successful tocolysis in 84% of the women (unsuccessful in 5.3%) Group B: Reduction of the fenoterol dose	[50]
	Bryophyllum 5% infusion *Bryophyllum* 50% trituratio	*in vivo*	Retrospective study: *B. pinnatum* vs. *B. pinnatum* + fenoterol Group A: *B. pinnatum* (n=89) Dose: infusion 580 mg/h followed by trituratio 200 mg/h p.o. Group B: *B. pinnatum* + fenoterol (n=81) Dose: infusion 580 mg/h; additionally after 2h fenoterol i.v. or p.o. followed by trituratio 200 mg/h p.o.	Altoghether, *B. pinnatum* alone showed a comparable outcome to the combination group. No side effects were reported.	[56]
	Bryophyllum 33% dilutio	*in vivo*	Evaluation of data from 1622 deliveries (from 1977 to 2000) Incidence of premature deliveries 1977-1983: fenoterol i.v. or p.o. (incidence of 6.2%) 1983-1990: *B. pinnatum* 33% dilutio, fenoterol i.v. or p.o. (half-dose given if combinated) 1990-2000: *B. pinnatum* 33% dilution Dose: 5 x 20 drops (each 4 h) or 20 drops each 1-2h	After 1983: Decrease of the incidence of premature deliveries to 1.07%	[57]
	Bryophyllum 5% ampoules	*in vivo*	Retrospective study: *B. pinnatum* vs. beta-agonists Pregnant women in premature labour n=134 (67 patients in each group) Dose: max. 600 mg/h i.v. for at least 48h or cessation of contractions Alternative if therapy is ineffective: parenteral beta-agonist followed by *B. pinnatum* 50% chewable tablets, 200mg/2 h on average	Maternal outcome Significant decrease of maternal adverse effects Less corticosteroids and antibiotics Similar prolongation of pregnancy in both groups Neonatal outcome Higher Apgar scores Lower oxygen use and morbidity	[58]

Tocolysis	*Bryophyllum* 50% chewable tablets	*in vivo*	Prospective, randomised study: *B. pinnatum* vs. Nifedipine Pregnant women in premature labour n=27 (n=14 patients in group A) 1) Change in bishop score: Visit 1 (0-1h) and visit 2 (4h) 2) Difference in the number of contractions/h: Visit 1 and visit 2 3) Change in blood pressure: Start (0h) and visit 2 4) Neonatal outcome (APGAR score) 5) Hospital stay Group A: *B. pinnatum* 50% p.o. Dose: 1 tablet each 15 min for 1 h, continue with 2 tablets each 6h for at least 48h Group B: Nifedipine Dose: 1 capsule 10 mg each 15 min for 1h, continue with alternating 1 capsule 60 mg and 30 mg each 12h for at least for 48h	1) No change between visit 1 and 2 No difference to Group B 2) Significant decrease of 5.1 contractions/h No difference to Group B 3) Significant increase in blood pressure No difference to Group B 4) No difference to Group B 5) Significant shortening of hospital stay compared to group B	[59]
	Aqueous fresh leaf extract	*in vitro*	Organ bath contractility experiment Myometrial biopsy during caesarean section n=14 (muscle strips n=85) 1) Effect on spontaneous contractions Conc.: 10^4 mg/L 2) Effect on oxytocin-stimulated contractions a) Conc.: 5 x 10^{-1} mg/L b) Conc.: 5 x 10^3 mg/L c) Conc.: 10^4 mg/L	1) Muscle relaxation: Reduction in AUC of 16% Increase of the frequency of 90% 2) a) Muscle relaxation: Reduction in AUC of <10% No change in amplitude b) Decrease of the frequency of 33% c) Muscle relaxation: Reduction in AUC of <20% Decrease in mean amplitude of 8%	[60]
	Leaf press juice	*in vitro*	Organ bath contractility experiment Myometrial biopsy during caesarean section (muscle strips) Effect on spontaneous contractions 1) AUC 2) Amplitude (intensity of the contractions) 3) Contraction frequency Conc. press juice: undiluted (2 µL added to the chamber) Conc. fractions: 1% - 10%, undiluted (2 µL added to the chamber)	1) Significant reduction to 82% after 1 application 2) Significant reduction to 78% after 2 applications 3) Significant fast increase to 128% after 1 application	[61]
	Flavonoid fraction			Undiluted fraction: 1) Significant reduction to 51% after 1 application 2) Significant reduction to 70% after 1 application 3) Significant fast increase to 557% after 1 application	
	Bufadienolide fraction			1) No significant change 2) No significant change 3) Fast increase	
	Fraction containing cinnamic acid derivatives				

Tocolysis	Leaf press juice	*in vitro*	1) Effect on oxytocin-induced increase in $[Ca^{2+}]_i$ a) Human myometrial hTERT-C3 cells (loaded with Fura-2) b) Human myometrial M11 cells 2) Effect on oxytocin-induced increase in $[Ca^{2+}]_i$, Ca^{2+}-free condition Human myometrial hTERT-C3 cells (loaded with Fura-2) 3) Effect on voltage-dependent increase of $[Ca^{2+}]_i$ SH-SY5Y human neuroblastoma cells Conc.: 0.1-2% (v/v)	1) a) Dose-dependent inhibition of $[Ca^{2+}]_i$ increase IC_{50} = 0.94% b) Weaker inhibition 2) Significant inhibition 3) No inhibition, but slower increase than control cells	[62]
Overactive bladder	*Bryophyllum* 50% chewable tablets	*in vivo*	Prospective, double-blind randomised, placebo-controlled study Postmenopausal women n=20 (10 patients in each group) 8-Week treatment (5 visits during 15 weeks) Baseline, visit 3, 4, and 5: 2-day bladder diary QoL and OAB bother: KHQ ICIQ-OAB Dose: 3 x 2 blinded capsules per day	Micturition frequency/24h decreased: 9.5 ± 2.2 before and 7.8 ± 1.2 after Placebo: 9.3 ± 1.8 before and 9.1 ± 1.6 after QoL was comparable in both groups	[63]
	Leaf press juice	*in vitro*	Organ bath contractility experiment Porcine urinary bladders n=32 (detrusor muscle strips n=37) 1) Effect on the electrically induced muscle contractions 2) Effect on the carbachol pre-contracted muscle strips Conc.: Cumulative addition of 0.1, 0.5, 1, 2.5, 5, 10%	1) Significant max inhibition of 74.6 ± 10.2% with 5% 2) Significant max relaxation of 18.7 ± 3.7% with 10%	[64]
	Leaf press juice	*in vitro*	Organ bath contractility experiment Porcine urinary bladders n=35 Inhibitory effect on the electrically induced muscle contractility Conc. press juice: 2.5, 5, 10% Conc. flavonoid fraction: 0.1, 0.33, 0.4, 0.5, 0.64, 0.8, 1 mg/mL Conc. bufadienolide fraction:0.1, 0.5, 2, 5, 15, 30, 40 µg/mL Conc. polar fraction: 0.5, 1, 2, 3, 4, 5 mg/mL	Inhibition of 41.4% with a conc. of 10%	[65]
	Flavonoid fraction			Significant inhibition of 78.7% with a conc. of 1 mg/mL	
	Bufadienolide fraction			No inhibition	
	Polar fraction (L-malic acid, sugars)			Inhibition of 84.5% due to lowering of pH in the chamber	

Sleep disorders	*Bryophyllum* 50% chewable tablets	*in vivo*	Prospective, multi-centre, observational study Pregnant women n=49 Questionnaires before and at the end of the 14-day treatment: Sleep quality: Adapted Pittsburgh Sleep Quality Index (PSQI) Daytime sleepiness: Epworth Sleeping Scale (ESS) Fatigue: Fatigue Severity Scale (FSS) Dose: 3 - 8 tablets per day	Significant decrease of the number of wake-ups Significant improvement of the subjective quality of sleep Decrease of the daytime sleepiness No prolongation of the sleep duration No reduction in the time to fall asleep	[66]
Neurological disorder	Aqueous leaf extract	*in vivo*	Swiss mice 1) CNS depressant effects Pentobarbitone-induced sleep 2) Exploratory activity Hole-board method and evasion test 3) Muscle relaxant tests Chimney, traction, climbing, and inclined screen test 4) Anticonvulsant tests Strychnine- and picrotoxin-induced convulsions Conc. aqueous extract: 50, 100, 200 mg/kg p.o. Conc. methanolic extract: 100, 200, 400 mg/kg i.p.	1) Dose-dependent prolongation of the onset and duration of sleeping time 2) Reduction of head-dip Increase of mice remaining in box 3) Dose-dependent muscle relaxant effect 4) Minor anticonvulsant activity	[67]
	Methanolic fraction			1) Significant dose-dependent prolongation of the duration of sleeping time 3) Chimney test: Significant loss of coordination and muscle relaxant effect with 300 mg/kg 4) No significant protection against strychnine-induced convulsion	[68]
	Methanolic fraction	*in vivo*	Swiss mice Estimation of the brain GABA concentration Dose: 300 mg/mL i.p.	Significant increase in brain GABA concentration	
	Bersaldegenin-1,3,5-orthoacetate	*in vivo*	Mice Radar-monitored cage a) Dose: 0.1 - 0.5 mg/kg b) Dose: > 0.5 mg/kg	a) Strong sedative effect b) CNS related activites: paralysis, muscle contractions	[27]

2.1.7.2 Other activities

The various pharmacological activities of *B. pinnatum* have been investigated *in vitro* and *in vivo* (detailed information are provided in Table 3). Some of the described activities have already been used in traditional medicine, but until now only a few *in vivo* studies in humans have been reported.

Antileishmanial activity

Leishmaniases comprises several diseases that are caused by protozoan parasites from over 20 *Leishmania* species. The protozoa are transmitted by the bite of female phlebotomie sandflies.

B. pinnatum aqueous extract treatment in a cutaneous leishmaniasis patient demonstrated no further growth and a slight decrease in the active lesion. At the end of the 14-day treatment period, the toxicological parameters of the patient's serum were within the reference range [69]. In mice, the effect of an aqueous *B. pinnatum* extract was investigated after oral (by intragastric intubation), i.v., intraperitoneal (i.p.) and topical (by rubbing the lesion site) administration. The oral treatment was most effective and was able to prevent and delay the onset of lesion growth in a sustained manner. Additionally, after oral, i.p., or topical application, the parasite-specific antibody (IgG) titer was reduced to 20% compared with untreated mice [70]. Further investigations revealed that flavonoids possessed the antileishmanial activity. Quercitrin had the highest *in vitro* antileishmanial activity and low cytotoxicity. A quercetin diglycoside was the second most active flavonoid followed by its aglycone quercetin and kaempferol glycosides. It has been suggested that the aglycone quercetin is relevant to the antileishmanial activity [11,71]. Orally administered quercetin and quercetin glycosides were able to stop lesion growth in mice. An explanation for the similar activity *in vivo* may be the metabolism of these flavonoids, which produces the same active metabolites [72].

Antimicrobial activity

In vitro experiments have demonstrated the sensitivity of several tested bacteria and fungi to the hot water and methanolic extract as well as to flavonoids of *B. pinnatum* using the agar-well diffusion method (minimum inhibitory concentrations (MICs) are shown in Table 3). Further investigations revealed *α*-rhamnoisorobin and several flavonoids, including kaempferol glycosides, to be responsible for the antibacterial and antifungal activity of *B. pinnatum* [13,19,36,44,45].

Insecticidal activity

Different bufadienolides were tested in an *in vitro* bioassay using 3rd instar larvae of the silkworm. Larvae were cultured on an artificial diet and further put into petri dishes containing the test samples (added to 1 g of the diet). The mortality rate was determined after 24 hours. Bryophyllin A, bryophyllin C, and bersaldegenin-1,3,5-orthoacetate showed LD_{50} values of 3, 5 and 16 µg/g of diet, respectively, whereas bersaldegenin-1-acetate and bersaldegenin-3-acetate showed no insecticidal activity. These results suggest that the 1,3,5-orthoacetate moiety is essential for the insecticidal effect [24,73].

Anticancer and antitumor activity

An *in vitro* study demonstrated a dose-dependent inhibition of human cervical cancer cell growth using a *B. pinnatum* chloroform extract and a fraction containing steroidal glycosides, alkaloids, and steroids. The fraction was more potent than the extract and demonstrated pro-apoptotic activity. However, the extract had higher anti-HPV activity than the fraction [74].

In an earlier study, five bufadienolides were shown to possess anti-tumour promoting activity by inhibiting Epstein-Barr virus early antigen (EBV-EA) activation. These investigations indicated that the 1,3,5-orthoacetate moiety was important for the chemopreventive activity [75].

The effect of bufadienolides against several tumor cells was tested in an *in vitro* assay. Bryophyllin A showed a potent cytotoxicity in human lung carcinoma A-549 cells, KB cells, and colon HCT-8 tumor cells with ED_{50} values of 10, 14, and 30 ng/mL, respectively. Bersaldegenin-3-acetate mainly demonstrated an effect against HCT-8 cells (ED_{50} = 10 ng/mL) and bryophyllin B showed cytotoxicity against KB cells with an ED_{50} value of < 80 ng/mL [23,76].

Antiallergic and antiasthmatic effects

B. pinnatum leaf press juice produced an antihistaminic effect in guinea pigs ileum *in vitro* and was able to prevent histamine-induced bronchoconstriction in guinea pigs *in vivo*. Flavonoids are hypothesized to be responsible for the selective and competitive inhibition of the H_1 receptor [43]. In addition, aqueous leaf extracts demonstrated an antiasthmatic effect by successfully protecting guinea pigs from histamine-induced preconvulsive dypsnoea. The reduction of coughing bouts in guinea pigs treated with this extract confirmed its antitussive properties [40].

Furthermore, mice were treated with *B. pinnatum* aqueous leaf extracts during a 14-day sensitisation (ovalbumin, OVA) period. Daily oral treatment with the leaf extract protected all mice from fatal anaphylactic shock. Quercitrin had a protective effect in 75% of the animals and appears to be important for the antianaphylactic effect of the extract. Furthermore, the

aqueous leaf extract reduced eosinophilia as well as IL-5, IL-10 and TNF-α cytokine production [77]. In addition, the aqueous extract of *B. pinnatum* and quercetin, but not quercitrin, inhibited the development of allergic airway inflammation and airway hyperresponsiveness in mice. It is assumed that the inhibition of mast cell degranulation and reduction of TNF-α levels are involved in the antiallergic effect [78].

Antiulcer activity

The pretreatment of guinea pigs with leaf press juice was not able to prevent the development of histamine-induced ulcerations [43]. However, the methanolic fraction of *B. pinnatum* was demonstrated to possess antiulcer activity in rats. The development of different types of acute gastric ulcers was significantly inhibited after pretreatment. Additionally, the healing of acetic acid-induced gastric ulcers was improved [42,79].

Antinociceptive/analgesic activity

The antinociceptive activity of *B. pinnatum* has been demonstrated *in vivo*. Using a thermal test method, mice were treated with aqueous extract prior to exposure to a heat-induced nociceptive pain stimulus (hot plate). A chemical antinociceptive method observed the triggered abdominal contractions in mice after i.p. injection of 3% acetic acid. Both methods demonstrated that the aqueous extract provided significant protection against the nociceptive stimulus compared with diclofenac [46]. In addition, the analgesic potential of the aqueous extract, methanolic fraction and also of a steroidal compound in *B. pinnatum* was examined. Using the chemical method described above, the extract and stigmast-4,20(21),23-trien-3-one demonstrated a significant reduction in the number of contractions by 80.16% and 75.72%, respectively. In addition, also the methanolic fraction showed a significant reduction [34,68].

Hepatoprotective activity

In folk medicine, jaundice has been treated successfully with *B. pinnatum* leaf press juice. Therefore, the hepatoprotective effect of a concentrated press juice and an ethanolic extract of the marc (left after expressing the juice) were examined *in vitro* and *in vivo*. The leaf press juice was more potent in rat hepatocytes as well as in the rat model. An important discovery was a decrease in elevated serum bilirubin levels by the juice and a decrease of serum glutamyl pyruvate transaminase (SGPT) levels by the juice and the ethanolic extract, which is relevant for acute jaundice treatment [41].

Antiurolithic activity

Many individuals suffer from urolithiasis. Therefore, the antiurolithic activity of an ethanolic extract of *B. pinnatum* was assessed. Fresh urine from a man was mixed with different concentrations of the extract before sodium oxalate solution was added to induce crystallisation. A concentration-dependent increase of the number of crystals was observed. However, the size of calcium oxalate monohydrate (COM) crystals was significantly reduced and even disappeared totally with an extract concentration of 100 mg/mL. Further, the formation of calcium oxalate dihydrate (COD) crystals was promoted rather than COM. COD crystals are less urolithic than COM and therefore the result is promising for treatment of urolithiasis [80].

Anti-inflammatory activity

To determine the anti-inflammatory activity, the paw oedema method in Wistar rats was performed. The aqueous leaf extract significantly reduced paw oedema inflammation [46]. This result was confirmed in a second experiment revealing a significant reduction of acute inflammation by the aqueous extract and stigmast-4,20(21),23-trien-3-one of 87.3% and 84.5%, respectively. These results suggest the steroidal compound to be involved in the anti-inflammatory activity [34]. Furthermore, ear oedemata in Swiss albino mice were significantly inhibited by topical application of an ethanolic extract of *B. pinnatum* [15].

Antioxidant activity

Many flavonoids are known to possess an antioxidant effect. Therefore, the antioxidant activity of aqueous leaf extracts as well as of quercetin 3-O-*α*-L-arabinopyranosyl (1 → 2) *α*-L-rhamnopyranoside was measured. The plants used for ethanolic extracts were grown under three different lights. The light treatments with either white lamps, white lamps plus blue lamps or white lamps plus UV-A lamps had no significant influence on the total phenolic content of the produced extracts. Using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical method, the extract treated with additional blue lamps showed an improved antioxidant activity compared to the other two light treatments. Also a changed phenolic profile of this extract could be shown. Quercetin 3-O-*α*-L-arabinopyranosyl (1 → 2) *α*-L-rhamnopyranoside showed a powerful anti-oxidant activity with an EC_{50} value of 1.41 µg/mL [81].

Antidiabetic activity

An *in vivo* study in diabetic and normoglycaemic Wistar rats demonstrated a high hypoglycaemic effect after oral treatment with aqueous leaf extract [45].

Wound-healing activity

The ethanolic leaf extract of *B. pinnatum* reduced wound areas 86.3% in Sprague Dawley rats. A significant increase in wound contractions and a decrease in oedema at the wound site were also observed [82].

Table 3. Investigation of different pharmacological activities of *B. pinnatum*.

Pharmacological activity	**Preparation/substance**	**Bioassay/study design**		**Effect**	
Antileishmanial activity	Aqueous leaf extract	*in vivo*	Male patient n=1: Dose: 2 x 15 g p.o. during 14 days	Growth inhibition of lesion Slight decrease of lesion	[69]
	Aqueous leaf extract	*in vivo*	Infection in the foodpad of BALB/c mice with *L. amazonensis* promastigotes Effect on lesion growth: 1) Treatment on day 7 after infection, repeated weekly 2) Treatment on day 7 after infection, repeated daily 3) Treatment on day 34 after infection, repeated daily Effect on parasite growth: Quantification by limiting dilution analysis (LDA) 4) Treatment on day 30 after infection, repeated daily Suppression of IgG: 5) Treatment on day 30 after infection, repeated daily a) Dose: 4 mg/0.2 mL oral b) Dose: 2 mg/0.1 mL i.v. c) Dose: 8 mg/0.2 mL oral d) Dose: 7.5 mg topical e) Dose: 4 mg/0.1 mL i.p.	1) a) Decrease of lesion growth b) Onset of lesion growth was delayed 2) c) Long-lasting change of the foodpad size to normal 3) c) Preventing effect on lesion development. 4) c) Effective suppression of parasite growth d) Reduction of lesion size and parasite load 5) a) d) e) Reduced IgG titer to 20%	[70]
	Quercitrin	1) *in vitro* 2) *in vivo*	1) Antiamastigote activity: Mouse peritoneal macrophages infected with promastigotes Conc.: until 100 µg/mL 2) Subcutaneous infection in the ear of BALB/c mice with *L. amazonensis* promastigotes Dose: extract: 320 mg/kg Dose: flavonoids: 16 mg/kg	1) 93.9% inhibition of amastigotes IC_{50} ≈ 1 µg/mL (2 µM) IC_{50} ≈ 8 µg/mL (18µM) 2) 57% reduction of the parasite burden	[11] [71] [72]
	Quercetin 3-O-*α*-L-arabinopyranosyl (1 → 2) *α*-L-rhamnopyranoside			1) 60.5% inhibition of amastigotes IC_{50} ≈ 45 µg/mL (78 µM) 2) 65% reduction of the parasite burden	
	Kapinnatoside			1) 39.2% inhibition of amastigotes IC_{50} > 100 µg/mL (> 177 µM)	

Antileishmanial activity	4',5-dihydroxy-3',8-dimethoxyflavone 7-O-*β*-D-glucopyranoside		1) Antiamastigote activity: Mouse peritoneal macrophages infected with promastigotes Conc.: until 100 µg/mL 2) Subcutaneous infection in the ear of BALB/c mice with *L. amazonensis* promastigotes Dose: extract: 320 mg/kg Dose: flavonoids: 16 mg/kg	1) 34.9% inhibition of amastigotes IC_{50} > 100 µg/mL (> 203 µM)	
	Quercetin			1) IC_{50} > 100 µg/mL (> 331 µM) 2) 76% reduction of the parasite burden	
	Aqueous leaf extract (lyophilised)			2) 70% reduction of the parasite burden	
Antimicrobial activity	Hot water extract	*in vitro*	Agar-well diffusion method a) A. niger b) *B. subtilis* c) *C. albicans* d) *C. parapsilosis* e) *C. neoformans* f) *E. coli* g) *K.pneumoniae* h) *P. vulgaris* i) *P. aeruginosa* j) *S. aureus* k) *S. dysenteriae* l) *S. typhi* Conc. extract: 2.5-20.0 mg/mL, 25 mg/mL (MeOH extract 60%) Conc. isolated compounds: 6.25 - 100 mg/mL Conc. *α*-rhamnoisorobin: 0.25 - 1024 µg/mL	MIC [µg/mL]: a) no inhibition, b) 33.11, c)–i) no inhibition, j) 37.81, k)-l) no inhibition	[45]
	Methanolic extract			MIC [µg/mL]: a) no inhibition, b) 87.10, c)–i) no inhibition, j) 36.31, k)-l) no inhibition	
	Methanolic extract (60%)			MIC [mg/mL]: b) 4.38, c) no inhibition, f) 2.19, g) no inhibition, h) 2.19, i) no inhibition, j) 4.38, k) 2.19	[44]
	5' Methyl 4',5,7 trihydroxyl flavone			MIC [mg/mL]: a) 100, c) 0.25, f) no inhibition, g) 5.0, i) 2.5, j) 12.5	[19]
	4',3,5,7 Tetrahydroxy 5-methyl 5'-propenamine anthocyanidines			MIC [mg/mL]: a) 25, c) 50, f) 100, g) 50, i) 12.5, j) 25	[36]
	1-Ethanamino 7 hex-1-yne-5'-one phenanthrene			MIC [mg/mL]: a) 100, c) 100, f) 100, g) 100, i) 50, j) 50	
	α-Rhamnoisorobin			MIC [µg/mL]: c) 1, d) 2, e) 2, g) 1, i) 1, j) 2, k) 1	[13]
Insecticidal activity	Bryophyllin A	*in vivo*	Bioassay with *Bombyx mori* larvae (3rd instar silkworm larva) Larvae were treated in petri dishes containing the test sample Mortality rate after 24 h was determined	LD_{50} = 3 µg/g	[24] [73]
	Bryophyllin C			LD_{50} = 5 µg/g	
	Bersaldegenin-3-acetate			No activity	
	Bersaldegenin-1,3,4-orthoacetate			LD_{50} = 16 µg/g	
	Bersaldegenin-1-acetate			No activity	

Anticancer and antitumor activity	Chloroform crude extract	*in vitro*	1) MTT test using human cervical cancer cells a) Conc.: 100 µg/mL b) Increasing concentration to examine cell viability at 24 h 2) Assessment of API-specific DNA binding by electrophoretic mobility shift assay (EMSA) 3) Anti-HPV and pro-apoptotic activity in cervical cancer cells	1) a) 30% inhibition of cell growth b) IC_{50} = 552 µg/mL 2) Strong inhibition of AP1 activity 3) Strong suppression of HPV18 expression	[74]
	Fraction (steroidal glycosides, alkaloids, steroids)			1) a) 55% inhibition of cell growth b) IC_{50} = 91 µg/mL 2) Inhibition of AP1 activity 3) Suppression of HPV18 expression Strong induction of apoptosis	
	Bryophyllin A	*in vitro*	Inhibitory test on EBV-EA activation in Raji cells Conc. bufadienolides: 0.16 - 100 µM	IC_{50} = 0.4 µM	[75]
	Bryophyllin C			IC_{50} = 1.6 µM	
	Bersaldegenin-3-acetate			IC_{50} = 3.0 µM	
	Bersaldegenin-1,3,5-orthoacetate			Not determined due to cytotoxicity over 4 µM	
	Bryophyllin A	*in vitro*	Cytotoxicity against tumor cells 1) KB cells 2) Human lung carcinoma A-549 3) Colon HCT-8 tumor cells 4) P-388 5) L-1210	1) ED_{50} = 14 ng/mL 2) ED_{50} = 10 ng/mL 3) ED_{50} = 30 ng/mL 4) and 5) ED_{50} = >4 µg/mL	[23] [76]
	Bryophyllin B			1) ED_{50} = <80 ng/mL	
	Bersaldegenin-3-acetate			1) ED_{50} = <40 ng/mL 2) ED_{50} = 40 ng/mL 3) ED_{50} = 10 ng/mL 4) and 5) ED_{50} = >4 µg/mL	
Antiallergic, antiasthmatic activity	Leaf press juice	1) *in vitro* 2) *in vivo*	1) Organ bath experiments with Guinea pig ileum Pretreatment with agonists: Histamine, Ach, KCl, and $BaCl_2$ Conc. leaf press juice: 50, 100 mg/mL Conc. flavonoid fraction: 0.15 mg/mL 2) Guinea pigs Protection against bronchoconstriction evoked by histamine Dose leaf press juice: 4 mL/kg	1) Inhibition of histamine-induced contractions No effect on the stimulation by Ach, KCl, $BaCl_2$ 2) Short protection from death by asphyxia	[43]
	Flavonoid fraction			1) Inhibition of histamine-induced contractions No effect on the stimulation by Ach, KCl, $BaCl_2$	

Antiallergic, antiasthmatic activity	Aqueous leaf extract (dried)	*in vivo*	1) OVA-sensitised guinea pigs Histamine exposition after 21 day-treatment until preconvulsive dyspnoea (duration) 2) Citric acid aerosol exposed guinea pigs a) Dose: 200 mg/kg b) Dose: 400 mg/kg	1) a) Preconvulsion time is extended by 23.2% Significant reduction of mucus viscosity b) Preconvulsion time is extended by 40.8% Significant reduction of mucus viscosity 2) a) Reduction of cough bouts to 63% b) Reduction of cough bouts to 49.3%	[40]
	Aqueous leaf extract	1) *in vivo* 2) *in vitro* 3) *in vitro*	1) Ovalbumin-hypersensitised BALB/c mice, challenge on day 14 Protective effect on fatal anaphylactic shock: Daily treatment during hypersensitisation a) Dose aqueous extract: 400 mg/kg p.o. b) Dose aqueous extract: 200 mg/kg i.p. c) Dose quercitrin: 20 mg/kg p.o Single dose 3h before ovalbumin (OVA) challenge d) Dose aqueous extract: 200 mg/kg i.p. 2) ELISA, OVA-specific IgE in mouse sera after treatment 3) T cell proliferation and cytokine production	1) a) Protection of 100% of the animals from fatalvvshock b) Protection of 80% of the animals from fatal shock c) Protection of 75% of the animals from fatal shock d) Protection of 60% of the animals from fatal shock 2) Prevention of eosinophilia and OVA-specific IgE 3) Reduced levels of IL-5, Il-10 and TNF-α (more pronounced by i.p.)	[77] [78]
	Quercitrin			1) IC_{50} > 100 µg/mL (> 331 µM) 2) 76% reduction of the parasite burden	
	Aqueous extract	1) *in vitro* 2) *in vivo* 3) *in vitro* 4) *in vivo* *5) in vivo*	1) Mast cell degranulation Bone marrow-derived mast cells from BALB/c mice Conc. aqueous extract: 250 - 1000 µg/mL Conc. flavonoids: 25 - 100 µg/mL 2) Antigen sensitization and challenge Mice sensitized by i.p. and nebulised OVA Dose aqueous extract: 400 mg/kg Dose flavonoids: 30 mg/mL 3) Antigen-specific immunoglobulin and cytokine ELISA Levels of IL-5, IL-13, TNF and IFN-γ in supernatants of BMMC cultures or in the bronchoalveolar (BAL) fluid 4) Cell counts in BAL fluid using Hemacolor staining kit 5) Airway reactivity Mice inhaled methacholine (MCh)	1) Significant decrease of degranulation and TNF 2) Reduced levels of different immunogloblins 3) Reduced levels of IL-5, IL-13, TNF, but not of IFN-γ 4) Significant reduction of lymphocytes and eosinophils 5) Significant reduction of airway reactivity to MCh	[78]
	Quercetin			1) Significant decrease of degranulation and TNF, IL-6 2) Reduced levels of different immunogloblins 3) Reduced levels of IL-5, IL-13, TNF, but not of IFN-γ 4) Significant reduction of lymphocytes and eosinophils 5) Significant reduction of airway reactivity to MCh	
	Quercitrin			1) No effect on degranulation, no decrease of cytokines 2) No reduction of immunoglobulin levels 3) No effect on levels of IL-5, IL-13, TNF, and IFN-γ 4) No effect on airway inflammation 5) No effect on airway reactivity	

Antiulcer activity	Leaf press juice	*in vivo*	Guinea pigs Histamine-induced gastric ulcer Dose: pretreatment with 4 mL/kg	No protection against the development of ulcer	[43]
	Methanolic extract	*in vivo*	1) Pretreatment of Charles-Foster rats a) ASA-, indomethacin-, serotonin-, ethanol induced ulcer b) Reserpine- and stress-induced lesions c) ASA-induced ulcer in pylorus-ligated rats 2) Acetic acid-induced ulcer in rats 3) Histamine-induced duodenal ulcers in guinea pigs Dose: pretreatment with 100 or 300 mg/kg i.p.	1) a) Inhibitory effect on ulcer formation b) Protection against the development of ulcer c) Protection against the development of ulcer 2) Promotion of wound healing 3) Inhibitory effect on ulcer formation	[42]
	Methanolic extract	*in vivo*	Wistar rats 1) Indomethacin-induced gastric ulcer 2) Basal and histamine-induced gastric acid secretion Dose: pretreatment with 10, 20, 40 mg/kg i.p.	1) Dose-dependent inhibition of ulceration 2) Decrease of gastric acid secrection	[79]
Antinociceptive/ analgesic activity	Aqueous leaf extract	*in vivo*	1) Hot-plate test method in Balb/c mice 2) a) Acetic acid test method in Balb/c mice Dose: 50-400 mg/kg i.p.	1) Extended reaction time to the nociceptive stimulus 2) a) Significant decrease of contractions	[46]
	Methanolic fraction	*in vivo*	Acetic acid test method in Swiss albino mice Dose fraction: 100, 200, 300 mg/kg i.p. Dose extract: 400 mg/kg i.p. Dose substance: 300 mg/kg i.p.	Significant decrease of contractions	[68]
	Aqueous leaf extract			Significant decrease of contractions of 80.16%	[43]
	Stigmast-4,20(21),23-trien-3-one			Significant decrease of contractions of 75.72%	
Hepatoprotective activity	Leaf press juice (concentrated)	1) *in vitro* 2) *in vivo*	Induction of hepatotoxicity by CCl_4 1) Rat hepatocytes, Trypan blue exclusion method of cell viability Level determination of GOT and GPT 2) Wistar rats Level determination of SGOT, SGPT, SALP, and SBLN Conc.: 100 mg/mL	1) Decrease of GOT and GPT levels by 55.6% and 69.6%, respectively 2) Decrease of SGOT, SGPT, SALP, and SBLN levels by 51.7, 92.5, 72.5, and 105.5%, respectively	[41]
	Ethanolic extract of the marc			1) Decrease of GOT and GPT levels by 36.5% and 38.6%, respectively 2) Decrease of SGOT, SGPT, SALP, and SBLN levels by 29.5, 81.4, 45.8, and 49.0%, respectively	

Antiurolithic activity	Ethanolic extract	*in vitro*	Crystallisation assay with urine obtained from a man Urine mixed with the extract and sodium oxalate solution a) Crystallisation process (number of crystals) b) Size of calcium oxalate monohydrate (COM) c) Size of calcium oxalate dihydrate (COD) Conc.: 1, 4, 8, 16, 32, 64, 80, 100 mg/mL	a) Concentration-dependent increase b) Significant reduction with 64-100 mg/mL Totally inhibited formation with 100 mg/mL c) Promoted formation of COD	[80]
Anti-inflammatory activity	Aqueous leaf extract	*in vivo*	Paw oedema method in Wistar rats (induced by fresh egg albumin) Dose extract: pretreatment 50, 100, 200, 400 mg/kg p.o.	Significant reduction of acute inflammation	[46]
	Aqueous leaf extract	*in vivo*	Paw oedema method in Wistar rats (induced by carrageenan) Determination of acute inflammation compared to diclofenac Dose extract: pretreatment 400 mg/kg p.o. Dose substance: pretreatment 300 mg/kg p.o.	Signifcant reduction of acute inflammation by 87.29%	[34]
	Stigmast-4,20(21),23-trien-3-one			Signifcant reduction of acute inflammation by 84.45%	
	Ethanolic extract	*in vivo*	Ear oedema in Swiss albino mice induced by: 1) Croton oil single application 2) Arachidonic acid-induced ear oedema 3) Phenol 4) Ethyl phenylpropiolate 5) Capsaicin Determination of inhibition of ear oedema Dose: pretreament with 0.1, 0.5, 1 mg/ear	1) Significant inhibition of 55% (0.5 mg/ear) 2) Significant inhibition of 42% (0.1 mg/ear) 3) Significant inhibition of 80% (0.1 mg/ear) 4) Significant inhibition of 43% (0.1 mg/ear) 5) Significant inhibition of 75% (0.1 mg/ear)	[15]
Antioxidant activity	Aqueous leaf extract	*in vitro*	1) Folin-Ciocalteau method - Determination of phenolic content 2) DPPH free-radical method - Determination of antioxidant activity Antioxidant activity compared to the white-light treatment a) Extract from plants grown under white lamps plus blue lamps b) Extract from plants grown under white lamps plus UV-A lamps c) Quercetin 3-O-*α*-L-arabinopyranosyl (1 → 2) *α*-L rhamnopyranoside Conc.: 0.5 - 250 µg/mL	1) No significant differences 2) a) Significantly improved antioxidative activity b) No difference c) ED_{50} = 1.41 µg/mL	[81]
	Quercetin 3-O-*α*-L-arabinopyranosyl (1 → 2) *α*-L-rhamnopyranoside				

Antidiabetic activity	Aqueous leaf extract	*in vivo*	Wistar rats Diabetes was induced by injection of streptozotocin Blood glucose conc. measured 0, 2, 4, and 8 h after administration Dose: 25 - 800 mg/kg p.o.	Significant reduction of blood glucose concentration Highes hypoglycaemic effect 2-4 h after administration	[46]
Wound-healing activity	Ethanolic extract	*in vivo*	Excision wound model in Sprague Dawley rats Dose: 100 mg/kg (topical application until day 11)	86.3% reduction in the wound area Significant increase of wound contraction Significant decrease in oedema	[82]

2.1.7.3 Tolerability studies

Bryophyllum pinnatum may be an excellent alternative therapy option due to the good tolerability (detailed information are provided in Table 4).

The administration of *B. pinnatum* 5% i.v. and 50% p.o. to induce tocolysis in women showed less side effects than betamimetics. Specifically, palpitation and dyspnea were significantly less observed due to the lack of effect on β_1-adrenoceptors [58]. In addition, the treatment of 14 pregnant women (*Bryophyllum* group) with *B. pinnatum* 50% chewable tablets demonstrated no side effects, which were assigned to the medication [59]. A further study revealed no significant difference in observed side effects. One woman treated with *B. pinnatum* 50% chewable tablets suffered from diarrhoea and dysentery, maybe due to lactose intolerance, and a second woman had an exanthema of the face and upper thorax [63].

In a longitudinal, prospective, randomised controlled animal study, the effect of the mother tincture (MT) 30% of *B. pinnatum* in pregnant Wistar rats was investigated. From day 0 of gestation, 60 rats were treated with the *B. pinnatum* MT or pure vehicle. Two control groups, C1 and C2, received an equivalent to the usual daily dose and 25 x the maximum daily dose of vehicle, respectively. Groups B1, B2, B3, and B4 received every day 1, 25, 50, and 100 x the maximum daily dose of MT, respectively. After 20 days of treatment, weight gain (excluding foetal and placental weight) was higher in group B4 than in groups B1, C2, and B2. However, the dams in group C1 were heavier than those in group B2. No maternal or foetal deaths, no differences in implantations and resorptions, no differences in the number and weight of foetuses and placentas were observed. External foetal abnormalities were not observed in groups B1-B4 [83].

Table 4. Tolerability studies of *B. pinnatum*.

Preparation	**Bioassay/study design**		**Effect**	
Bryophyllum 5% ampoules	*in vivo*	Retrospective study: *B. pinnatum* vs. beta-agonists Pregnant women in premature labour n=134 (Detailed information about the therapy are provided in Table 2)	Significantly less palpitation and dyspnea	[58]
Bryophyllum 50% chewable tablets	*in vivo*	Prospective, randomised study: *B. pinnatum* vs. Nifedipine Pregnant women in premature labour n=27 (Detailed information about the therapy are provided in Table 2)	No side effects were observed concerning the *B. pinnatum* therapy	[59]
Bryophyllum 50% chewable tablets	*in vivo*	Prospective, double-blind randomised, placebo-controlled study Postmenopausal women n=20 (Detailed information about the therapy are provided in Table 2)	Diarrhoea and dysentery, maybe caused by lactose intolerance (n=1) Exanthema of the face and upper thorax (n=1) No significant difference compared with placebo	[63]
Bryophyllum 30% mother tincture (MT)	*in vivo*	Longitudinal, prospective, randomised controlled study Pregnant Wistar EPM-1 rats n = 60 (10 rats in each group) 1) Weight gain (fetal and placental weight excluded) 2) Maternal and fetal mortality 3) Number of implantations and resorptions 4) Number and weight of fetuses and placentas 5) Major external fetal malformations Daily dose for 20 days (day 0 of gestation until laparotomy): B1: Usual max. daily dose (0.067 mL/kg) B2, B3, B4: 25 , 50, and 100 x the max. daily dose, respectively C1, C2: Equivalent to 1 and 25 x daily dose of vehicle, respectively	1) Dams in group B4 were significantly heavier than in groups B1, C2, and B2 Daems in group C1 were heavier than in group B2 2) No differences 3) No differences 4) No differences 5) No differences in the 4 dosage groups	[83]

2.1.7.4 Toxicity studies

B. pinnatum is well tolerated in patients. However, the toxicity of other *Bryophyllum* species has been reported based on the contained bufadienolides. A toxic effect of *B. pinnatum* in human is not expected due to the small amounts of bufadienolides.

The toxicity to cattle has been documented in earlier studies. A study was conducted including two claves, which were treated with flower heads of *B. pinnatum*. Clinical parameters were examined after administration of 20 g/kg by stomach tube. Five hours after dosing they became depressed and had rumen stasis as well as anorexia. The first calf died after 9 h due to dyspnoea, tachycardia, and exceeded heart rate. The second calf had diarrhoea for a long time until it died after 15.5 h. This study demonstrated a correlation between bufadienolides and the toxic effect in cattle [25].

An acute toxicity study was performed with a total of 25 mice, which were fed with either *B. pinnatum* methanolic extract, or distilled water. During 24 hours, the mice were observed and the mortality was noted. A dose of 25 mg/mL was found to be optimal because neither death nor side effects were observed, but the treatment with 200 mg/kg was lethal for 100% of the mice [79]. A similar study was perfomed including Swiss albino mice. Intraperitoneal treatment with an aqueous and methanolic extract and showed LD_{50} values in mice of 957 and 1159 mg/kg, respectively. Oral doses showed no toxicity up to 3 g/kg in mice and rats [84]. In addition, intraperitoneally administered methanolic fraction showed no death up to 2500 mg/mL in mice, but their behaviour changed with concentrations > 100 mg/mL [68].

The cardiotoxic activity of bersaldegenin-1,3,5-orthoacetate was investigated *in vitro* using isolated rabbit and guinea pig hearts. A strong positiv inotropic effect was shown [22].

Table 5. Toxicity studies of *B. pinnatum*.

Toxicity study	Preparation/substance	Study design		Effect	
Acute toxicity study	Flower heads (minced)	*in vivo*	Two calves Determination of symptoms and mortality Dose: 20 g/kg (wet weight of plant)	After 5h: Depression, rumen stasis, anorexia After 9h: first claf died, dyspnoea, tachycardia, exceeded heart rate After 15.5h: second calf died, diarrhoea, tachycardia	[25]
	Methanolic extract	*in vivo*	25 mice Determination of mortality after 24h Dose: 10-200 mg/kg p.o.	No death and side effect with 25 mg/kg LD_{100} = 200 mg/kg	[79]
	Methanolic fraction	*in vivo*	Ten albino mice Determination of mortality after 24h Dose: Different concentrations, i.p.	No death < 2500 mg/kg	[68]
	Aqueous extract	*in vivo*	1) Swiss albino mice 2) Swiss albino rats Determination of mortalitiy after 24h a) Dose.: 350-2600 mg/kg i.p. b) Dose.: 500-3000 mg/kg p.o.	1) a) LD_{50} = 957.02 mg/kg b) Non-toxic up to 3 g/kg 2) a) LD_{50} = 1064.21 mg/kg b) Non-toxic up to 3 g/kg	[84]
	Methanolic extract			1) a) LD_{50} = 1159.03 mg/kg b) Non-toxic up to 3 g/kg 2) a) LD_{50} = 1459.69 mg/kg b) Non-toxic up to 3 g/kg	
Cardiotoxic activity	Bersaldegenin-1,3,5-orthoacetate	*in vitro*	1) Rabbit and guinea pig hearts 2) a) Rabbit papillary muscle b) Guinea pig atria	1) Strong inotropic effect 2) a) Inotropic effect above a dose of 10^{-6} M b) Inotropic effect above a dose of 3×10^{-7} M	[22]

2.2 Human urinary bladder

2.2.1 Bladder anatomy

The urinary bladder is a musculomembranous organ in the pelvis. It is located above and behind the pubic bone. The position varies during empty and full bladder conditions and is also dependent on the condition of the rectum.

The urinary bladder is composed of four surfaces: the superior, posterior, and two inferolateral surfaces. The superior surface is triangular, covered by peritoneum and slightly arched in the empty state. The posterior surface is covered by peritoneum in its superior region and the inferior region is covered by the endopelvic fascia of the rectovesical septum. The downward directed inferolateral surfaces point at the apex and are fully covered by endopelvic fascia (Fig. 16).

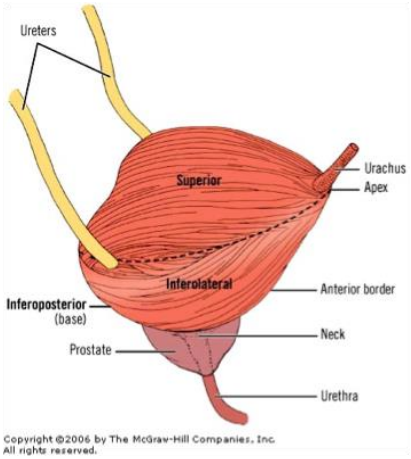

Figure 16. Lateral view of the urinary bladder [85].

Additionally, the bladder includes the apex, fundus, neck, and body (between apex and fundus). The apex is directed toward the upper margin of the symphysis and is connected to the umbilicus by the median umbilical ligament. The fundus is the inferior region of the posterior wall and includes the trigone, which is firmly connected to the muscular coat. The trigone is always smooth even when the bladder is empty and is delineated by the two orifices of the ureters and the bladder neck, which is the most inferior region and connects the urethra to the urinary bladder (Fig. 17) [87,88].

Please find the original figure on the homepage of the "Urology Care Foundation" (accessed June 2014): http://www.urologyhealth.org/urology/index.cfm?article=99

In the University Library of Basel you will find the printed version of this thesis with the slightly modified figure.

Figure 17. Urinary bladder anatomy: female and male. Anterior view of frontal section [86].

The bladder wall is composed of four layers: mucosa, submucosa, muscularis propria, and adventitia/serosa (Fig. 18). The mucosa represents the innermost layer and contains the urothelium, which lines the renal pelvis, ureters, bladder and a portion of the urethra. It consists of a basal cell layer (attached to the basement membrane), an intermediate layer and a superficial layer. The latter contains umbrella, cells including uroplakins, which act as a barrier by reducing the permeability of the urothelium. Tight junctions, as well as the glycosaminoglycan (GAG) layer, which covers the umbrella cells, are assumed to contribute

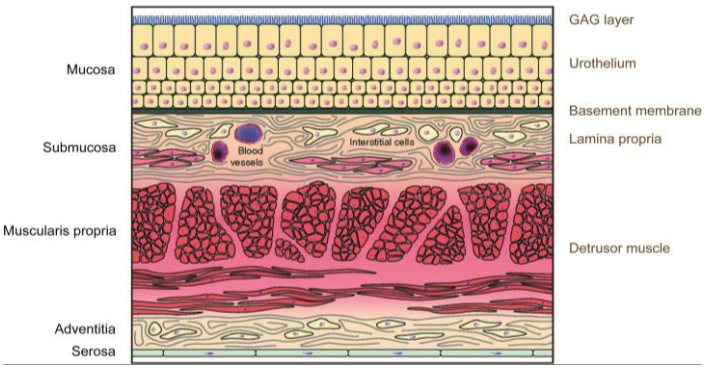

Figure 18. Bladder wall layers. Four major layers are included such as the mucosa, submucosa, muscularis propria, and adventitia/serosa. Modified from Birder et al. [89].

to the urothelial barrier function [89,90]. The submucosa is composed of areolar connective tissue, the lamina propria, which is cell rich and contains collagen and elastin for the organ's elasticity, as well as afferent nerve bundles, which are important sensors for the filling state of the bladder. The muscularis propria consists of the detrusor muscle and is responsible for the contraction and relaxation of the bladder. The outer layer is composed of the adventitia and serosa, which are connective tissues [87,89].

Detrusor muscle

Human detrusor muscle is composed of smooth muscle cells, which are classified into single-unit and multi-unit smooth muscles arranged into sheets or bundles. Detrusor muscle contractions are seldom spontaneous. They are induced by the ATP-dependent interaction between actin and myosin. The myosin-ATPase is regulated by myosin light chain kinase (MLCK), which is activated via the increased intracellular calcium concentrations $[Ca^{2+}]_i$. The adaption of the bladder during filling is explained by changes in cell length and not by a rearrangement of the cells [91].

2.2.2 Urinary bladder function

In the kidneys, the nephrons are responsible for the filtration of the blood to produce urine. Glomeruli produce 180 L of concentrated filtrate a day. Different resorption and secretion processes reduce the volume of the filtrate and alter its composition. Finally, the kidneys produce an average of 1.5 L urine a day that is excreted through the ureters into the bladder. The bladder is able to distend and store 400 - 600 mL of urine until voluntary micturition. Urine exits the bladder via the urethra, which is shorter in women (5 cm) than in men (20 cm) where the urethra is surrounded by the prostate and also releases spermatozoa [92].

Normal bladder function includes the storage and voiding/micturition phases, which are regulated by a neural control system in the brain and spinal cord, which includes the periaqueductal grey (PAG) and pontine micturition centre (PMC). The PMC switches between storage and voiding, depending on the complex afferent and efferent activity of the autonomic (parasympathetic and sympathetic) and somatic peripheral nervous systems [93-95].

2.2.2.1 Storage phase

Normal urine storage depends on spinal reflex mechanisms that activate sympathetic and somatic pathways to the urethral outlet and suppress the parasympathetic excitatory impulse to the urinary bladder [94]

The sympathetic storage reflex is triggered by afferent impulses from the activated stretch receptors via pelvic nerves to the spinal cord and further to the PMC. The efferent activity passes the hypogastric nerves and activates sympathetic nerves from T12-L2, which results in detrusor muscle relaxation and bladder neck contraction [95]. Therefore, the bladder is able to accommodate the increased pressure in the bladder until voiding. Additionally, the external urethral sphincter is regulated by the somatic nervous system via the pudendal nerve to guarantee some voluntary control over voiding and continence [96].

During the storage phase, the sympathetic postganglionic nerves release noradrenaline, which activates inhibitory β_3-adrenoceptors in the detrusor muscle. This consequently leads to bladder wall relaxation. Additionally, noradrenaline binds to excitatory α_1-adrenergic receptors in the urethra and the bladder neck to contract urethral smooth muscle [96,97]. The somatic axons in the pudendal nerve release ACh, which activates nicotinic cholinergic receptors and results in the contraction of the external urethral sphincter. These processes are relevant to maintaining continence during the storage phase.

In addition, the parasympathetic drive is suppressed and, as a consequence, acetylcholine (ACh) release is reduced to prevent detrusor muscle contraction. However, the afferent activity constantly increases during the bladder filling phase until micturition [93].

2.2.2.2 Micturition reflex

In adults, micturition is a voluntary process and is mediated by the activation of supraspinal pathways and inhibition of sympathetic pathways. Additionally, a prerequisite for voiding is the relaxation of the external urethral sphincter via the somatic pudendal nerves [95].

The reflex to void is initiated by two different pathways of small myelinated (Aδ) and unmyelinated (C) afferents. Aδ afferents send information about the state of bladder fullness from stretch receptors in the bladder wall via pelvic nerves to the sacral spinal cord (S2-S4) and also via the PAG matter to the PMC (located in the rostral pontine tegmentum). Therefore, the PMC is activated and efferents descend through the spinal cord to activate the sacral parasympathetic nerves leading to detrusor contractions and bladder voiding. Moreover, the inhibition of the lumbar sympathetic and sacral pudendal nerve nucleus results in the relaxation of the bladder neck and external urethral sphincter, respectively [98,99].

To induce micturition via transmitters, parasympathetic postganglionic axons in the pelvic nerves release cholinergic and non-adrenergic non-cholinergic transmitters (NANC) such as

ATP [96,97]. The cholinergic component of bladder control includes the release of ACh from parasympathetic nerves and release from a non-neurogenic source, which may be the urothelium [93]. ACh is the most important neurotransmitter responsible for bladder contraction and can induce micturition directly by acting on afferent nerves and indirectly by releasing other mediators [100]. Until micturition, the umbrella cells release ACh, nitric oxide (NO), and ATP, which alter the excitability of afferent fibres [101]. Furthermore, voiding is induced by NO release in the bladder outlet, which leads to relaxation [96].

Smooth muscle contractions depend on increased intracellular calcium concentrations $[Ca^{2+}]_i$. Calcium is able to enter the cytosol via L-type Ca^{2+} channels or through the release from the sarcoplasmic reticulum (SR) via IP_3 receptors, which is triggered by inositol-1,4,5-triphosphate (IP_3). Additionally, bladder stretching is also able to induce Ca^{2+} release from the SR. The SR can regulate the contraction via K^+ channels and Ca^{2+} influx to decrease $[Ca^{2+}]_i$.

The phosphorylation pathway is also important for contraction. Ca^{2+} complexes with calmodulin, which activates myosin light chain kinase (MLCK). Subsequently, MLCK phosphorylates the myosin light chain (MLC), which results in MLC-P and smooth muscle contraction [91]. The counteracting enzyme MLC-phosphatase dephosphorylates the MLC-P and induces muscle relaxation. In addition to the MLCK-pathway, the Rho-kinase (ROCK) pathway regulates detrusor contraction and is independent from Ca^{2+} increases. ROCK inhibits the MLC-phosphatase, sensitises the detrusor to calcium and induces contraction [96,102].

Various receptors can initiate the previously described pathways to induce bladder contraction.

Muscarinic acetylcholine receptors

Muscarinic acetylcholine receptors belong to the G-protein-coupled receptor family. In the human bladder, the excitatory M_1, M_3, and M_5 muscarinic receptors (coupled to $G_{q/11}$) and the inhibitory M_2 and M_4 subtypes (coupled to $G_{i/o}$) have been defined.

In the human detrusor, the M_2 subtype predominates, but M_3 receptor stimulation by ACh appears to be the most important for detrusor contraction. The activation of the receptor, and thus $G_{q/11}$, up-regulates phospholipase C (PLC) and leads to phosphoinositide hydrolysis, generating IP_3 and 1,2-diacylglycerine (DAG), which results in the influx of extracellular calcium as well as the release of Ca^{2+} from intracellular stores. This increased $[Ca^{2+}]_i$ leads to muscle contraction [96,103]. In addition, both the MLCK-dependent pathway and ROCK pathway are involved in the contraction mediated by muscarinic receptors. Therefore, the inhibition of MLCK and ROCK could also be a target to prevent bladder contraction (Fig. 19) [96,102].

The M_2 and M_4 receptors initiate an additional pathway. These receptors bind to pertussis toxin-sensitive $G_{i/o}$. and inhibit adenylyl cyclase (AC), inhibiting the cyclic AMP-induced relaxation [91].

In the urothelium, M_2 receptors are only expressed in the umbrella cells, whereas the M_3, M_4, and M_5 receptors are expressed throughout the urothelium and detrusor muscle cells. The activation of the urothelial muscarinic receptors by an agonist induces the release of ATP and the production of NO [96].

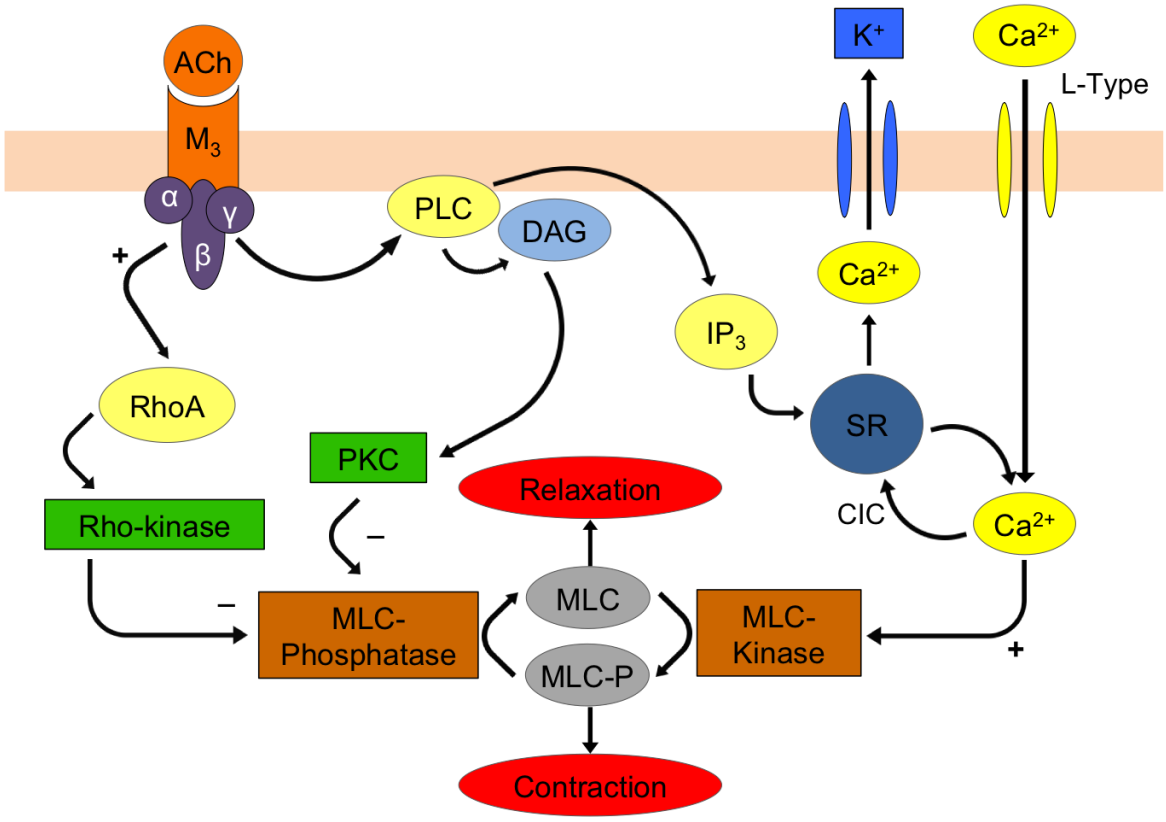

Figure 19. Detrusor muscle contraction induced by the activation of M_3 muscarinic receptor signalling. ACh, acetylcholine; PLC, phospholipase C; DAG, diacylglycerol; IP_3, inositol-trisphosphate; PKC, protein kinase C; MLC, myosin light chain; SR, sarcoplasmic reticulum; CIC, calcium-induced calcium release. The activation of M_3 receptor by ACh induces Ca^{2+} influx via L-type Ca^{2+} channels and calcium release from the SR. The Rock pathway and MLCK-dependent pathway are briefly illustrated. On the basis of Andersson et al. [91].

Adrenoceptors

Adrenoceptors are G-protein-coupled receptors, which are subdivided into α_1-, α_2-, β_1-, β_2-, and β_3-adrenoceptors and are targets of noradrenaline and adrenaline.

α-Adrenoceptors are primarily expressed in the bladder neck and urethra, but are only moderately expressed in the detrusor and bladder dome. α_1-Adrenoceptors are primarily expressed in men, specifically in the prostate, whereas α_2-adrenoceptors are primarily expressed in women. Stimulation of the α_1-adrenoceptors activates PLC, which leads to bladder contractility. α_2-Adrenoceptors inhibit the release of noradrenaline and induce bladder contraction via AC inhibition. Conversely, β-adrenoceptor stimulation induces bladder relaxation by activating AC and releasing cyclic AMP (cAMP), which further activates protein kinase A (PKA) [91,104].

2.3 Overactive bladder syndrome

2.3.1 Pathophysiology

Overactive bladder syndrome (OAB) is a symptomatic diagnosis that was defined by the International Continence Society (ICS) as urinary urgency, with or without urge incontinence, usually with frequency and nocturia, after the exclusion of urinary tract infection (UTI) or other obvious pathology [105]. Overactive bladder is a symptomatic syndrome without a definitive aetiology or pathophysiology. Urgency is the cornerstone symptom of OAB, which is defined as the sudden compelling desire to micturate that is difficult to delay.
In contrast, detrusor overactivity (DO) is diagnosed using urodynamic studies and includes involuntary detrusor contractions during the filling phase. Detrusor overactivity is categorised into idiopathic DO and neurogenic DO [106].
Several hypotheses have been proposed to explain the origin of OAB pathophysiology including the myogenic, neurogenic, and autonomy theories [107].

2.3.1.1 The myogenic theory

The myogenic theory suggests that there are changes in the properties of detrusor myocytes in DO patients, compared with normal detrusor myocytes. Partial denervation of the detrusor leads to an alteration of smooth muscle properties, including an increased spontaneous activity and electrical coupling between the smooth muscle cells. Therefore, excitation spreads to affect a larger region of the bladder wall and results in the continuous rise of intravesical pressure. Additionally, local contractions of some smooth muscles are caused by the stretching of parts of the bladder wall and the subsequently activated stretch-sensitive neurons and non-selective cation channels. The myogenic basis, which is relevant for instable bladder contractions, does not preclude an involvement of alterations in the neuronal pathways of the micturition reflex [108].

2.3.1.2 The neurogenic theory

The neurogenic theory assumes that damage to the brain that reduces suprapontine inhibition or to axonal pathways in the spinal cord can initiate primitive voiding reflexes, which leads to OAB.
Neurologic disorders in peripheral and central neural pathways may induce OAB. Possible alterations include a reduction in peripheral or central inhibition, an enhancement of excitatory transmission in the micturition reflex pathway, increased primary afferent input from the lower urinary tract (LUT), and emergence of bladder reflexes that are resistant to central inhibition [94]. Beside Aδ afferents, a second pathway is involved that is mediated by

C-fibres. C-fibres are primarily involved in pain sensation during disease. C afferents may induce the spinal segmental reflex pathway, which is responsible for detrusor contractions and neurogenic DO [99]. In addition, the inhibition of sympathetic innervation by surgical interruption or pharmacological treatment has been proven to reduce urethral outflow resistance, bladder capacity and increase the frequency and intensity of bladder contractions [109]. Patients suffering from neurologic diseases often exhibit OAB. Neurologic diseases are categorised into supraspinal (Parkinson's disease, cerebral palsy), spinal (spinal cord injury, spinal stenosis), suprasacral (detrusor sphincter dysynergia), peripheral (diabetes mellitus, herpes zoster), and mixed [95].

2.3.1.3 The autonomy theory

The autonomy theory attempts to describe the cause of overactive bladder because neither the myogenic nor the neurologic theory fully accounts for all recognised clinical and experimental findings. This new hypothesis is based on observations of muscle activation processes within the gastrointestinal tract. The autonomy theory suggests that the detrusor muscle is arranged into modules, which are active during the filling phase. A peripheral myovesical plexus, consisting of interstitial cells and intramural ganglia, controls these circumscribed areas of muscle. Intramural ganglia are able to receive inputs from neighbouring modules, pelvic organs, afferent collaterals, and interstitial cells. The activation of a single module results in micromotions, while the contraction of multiple modules produces macroscopic movements resulting in the contraction of the entire bladder. The synchronisation of all of the modules by the intramural nerve or interstitial cell networks may induce bladder voiding, as it occurs after CNS stimulation of the detrusor.
Therefore, this theory suggests that OAB is induced by an exaggerated symptomatic expression of peripheral autonomous activity [110].

2.3.2 Epidemiology

Prevalence
Overactive bladder affects millions of people and is frequently underreported. Patients often do not seek treatment because they do not expect any viable treatment options. Moreover, younger patients are often embarrassed by their condition and elderly patients consider OAB symptoms as a natural consequence of aging [111].
Several studies were performed based on the OAB definition provided by the ICS. The National Overactive Bladder Evaluation (NOBLE) program investigated the prevalence of OAB, with or without urge incontinence, in the United States adult population. The overall prevalence of OAB was 16.0% for men and 16.9% for women. Independent of age, men

were shown to suffer more from OAB without urge incontinence than women. In both genders, the ratio of OAB wet to OAB dry increased with age [112]. In 2005, the EPIC study (Sweden, Italy, Canada, Germany and United Kingdom), which included 19,165 individuals, was performed and the reported OAB prevalence rates were 12.8% in women and 10.8% in men [113]. In 2008, the worldwide OAB prevalence was estimated to be 10.7%, which is expected to rise to 20.1% in 2018 [114]. Although OAB prevalence rates are similar in women and men, there are gender differences that are observed in age-specific groups. Women have a higher prevalence of OAB symptoms before the age of 60, whereas men have a higher prevalence after age 60 [113].

The negative impact of urge and urge incontinence on the health-related quality of life (HRQL) is considered to be more significant compared with frequency and nocturia. Urge and urinary incontinence can influence patients' physical, social, and psychological well-being as well as sexual and everyday activities. However, OAB without incontinence also strongly impacts quality of life (QoL), depending on the number of voids during the day and nightly awakenings. A study in the US asked 919 participants about their OAB symptoms, depression symptoms, and sleep disturbance based on validated scales. The results confirmed that patients with incontinence had the highest impacted QoL. Patients suffering from urgency reported a higher QoL impact compared with those without. Additionally, frequency and nocturia had a negative influence on patients' QoL [111].

Estimated financial impact

The investigation into the financial impact of OAB for patients and public healthcare systems is a difficult task. The NOBLE program estimated economic costs based on questions about bladder symptoms, self-care use, treatment, work loss, and OAB-related health consequences (e.g., UTI, skin infections, and hospital stays) in 2000. In total, the estimated economic cost of OAB was $12.02 billion, including $9.17 and $2.85 billion for the community and institutions, respectively. Furthermore, the average costs per year are $110 for men and $410 for women. The costs per person increase with age [115].

Risk factors

Patients suffering from neurological diseases (e.g., multiple sclerosis, Parkinson's disease, and stroke) often develop OAB [116]. The risk factors for OAB may be different for women and men. The NOBLE study determined that women with an increased body mass index had a higher prevalence of wet OAB, however, this trend was not observed in men.

In general, diet and lifestyle factors are not associated with OAB. However, excessive beer and potato consumption consume had a negative influence on OAB prevalence.

2.3.3 OAB diagnosis

To obtain a reliable diagnosis, the recording of a urinary diary is a helpful tool to provide an overview of fluid intake, micturition numbers, and urine volume. Furthermore, it is necessary to exclude urinary tract infections, hematuria, cancer, residual urine, as well as neurological and metabolic diseases. The presence of urinary incontinence is determined using the hand-wash-test. A pad test is administered that provokes detrusor contraction while the patient washes their hands with cold water for one minute. This non-invasive method can confirm and quantify urine leakage; however, it is not mandatory for the diagnosis of OAB. Urodynamic examinations are recommended in patients with complex symptoms, neurological disorders and when conservative therapies have failed [117]. In women, it is important to include a gynaecological and obstetric history as well as former surgeries. Depending on the diagnosis, patients have different treatment options. The available treatment options alleviate OAB symptoms by decreasing urgency, reducing urinary urge incontinence, and increasing the voided volume [116].

2.3.4 Non-pharmacological therapies

The first steps of conservative treatments include changes in lifestyle, bladder training, and pelvic floor muscle exercises.

Patients should avoid caffeine, alcohol, and hot spices due to their irritating effects on the bladder mucosa. In obese patients, weight loss can improve urgency symptoms. Patients suffering from nocturia should avoid drinking fluids after 6:00 pm and void before going to sleep.

Bladder training helps to suppress involuntary detrusor contractions and can achieve long-term effects as long as the patients continue exercising. For timed micturition, one technique in the bladder re-education, patients are advised to void according to their longest micturition interval. Each week, the duration between voids is incrementally prolonged for 10 minutes until the patient is satisfied with the results. Additionally, pelvic floor exercises can improve symptoms by contracting and relaxing the muscles [116].

Another alternative therapy option may be acupuncture. It is worth to recommend to patients with motivation and interest in complementary and alternative medicine, to non-responders or patients with a contraindication to anticholinergic treatment. Acupuncture showed improved QoL and symptom scores compared with placebo (sham acupuncture) in a blinded randomised trail [118].

2.3.5 Pharmacological therapies

Several differently acting drugs are administered to patients for the treatment of OAB. Muscarinic receptor antagonists are the first-line pharmacotherapy for OAB syndrome and urinary incontinence. Besides, other drugs including calcium antagonists, serotonin and noradrenaline reuptake inhibitors, and estrogens as well as intravesical injection of botulinumtoxin are used to reduce OAB symptoms.

Muscarinic receptor antagonists

Antimuscarinics act during the storage phase and have almost no effect on voiding contractions [119]. Anticholinergics decrease the intensity of contractions as well as decrease frequency, improve bladder capacity, and reduce urgency.

Muscarinic receptors are expressed not only in the bladder but also in other parts of the body, such as the brain, salivary glands, heart muscle or intestine, which explains many of their side effects. Typical antimuscarinic drug side effects are dry eyes and mouth, constipation, tachycardia, blurred vision, heartburn, hot and flushed skin, and sedation. Before prescribing an anticholinergic drug, other diseases including renal and hepatic failure as well as polymedication have to be considered. All anticholinergic drugs expect trospium chloride and transdermal oxybutynin are metabolised by the cytochrome P450, which has to be considered due to possible interactions with other cytochrome P450 metabolised drugs. Specifically, elderly patients have to have their side effects well-controlled also due to possible confusion, deterioration of memory or delirium [96,120].

In the last years, anticholinergic drugs have become more tolerable and effective due to new galenicals, such as extended release formulations and M_3 receptor selectivity.

In Switzerland, the Swissmedic approved and well-characterised anticholinergic drugs are the following:

The unselective anticholinergics oxybutynin, tolterodine, fesoterodine, or trospium chloride as well as the competitive muscarinic M_3 selective receptor antagonists, such as solifenacin and darifenacin. An overview of the available antimuscarinic medicines is given in Table 6.

Table 6. Selectivity and dosage of antimuscarinics, approved in Switzerland, are shown [121,122].

Substance	Trade name Galenic form	Dosage	Receptor activity
Oxybutynin	Ditropan® Tab 5 mg Kentera® transdermal patch 3.9 mg/24 h Lyrinel® OROS Ret Tab 5 mg, 10 mg, 15 mg	3 x 5 mg (max 20 mg) 1 patch twice a week 1 x 5 - 20 mg	M_1-M_5
Tolterodine	Detrusitol® SR Ret Caps 2 mg, 4 mg	1 x 4 mg	M_1-M_5
Fesoterodine	Toviaz® Ret Tab 4 mg, 8 mg	1 x 4 - 8 mg	M_1-M_5
Trospium chloride	Spasmex® Tab 20 mg Spasmo-Urgenin® Neo Drag 20 mg	2 x 20 mg	M_1-M_5
Solifenacin	Vesicare® Tab 5 mg, 10 mg	1 x 5 - 10 mg	M_3
Darifenacin	Emselex® Ret Tab 7.5 mg, 15 mg	1 x 7.5 - 15 mg	M_3

OROS = osmotic release oral system; SR = slow release

Oxybutynin

Oxybutynin has various pharmacological properties, such as anticholinergic, local anaesthetic, direct muscle relaxant, and antihistaminic activities. It has a well-proven efficacy, but unfortunately, also has severe anticholinergic side effects. Oxybutynin is available as immediate release (IR) and extended release (ER) tablets and as transdermal patches. A large multi-centre, placebo-controlled study against tolterodine ER demonstrated that oxybutynin patch treatment caused the fewest anticholinergic side effects [120].

Tolterodine

Tolterodine has no selectivity for a receptor subtype, but has more affinity for the bladder muscarinic receptors than salivary gland receptors. The liver metabolises tolterodine into its metabolite, 5-HT, which has comparable activity. A large randomised, double-blind placebo-controlled trial demonstrated the efficacy and tolerability of tolterodine IR and ER, whereby the treatment with tolterodine ER once daily was more effective in reducing urge incontinence episodes and fewer patients were affected by dry mouth [123]. The therapeutic effects of tolterodine ER and oxybutynin patches demonstrated similar benefits for urge and mixed incontinence compared with placebo [120,124].

Fesoterodine

Fesoterodine is a prodrug that is also metabolised into 5-HT. Fesoterodine metabolism involves peripheral esterases and undergoes little hepatic metabolism [120]. A double-blind placebo-controlled trail investigated the effect of fesoterodine 8 mg in patients responding suboptimal to tolterodine ER 4 mg. After a 12-week treatment, urge incontinence was significantly improved, the micturition frequency during 24h was decreased, and fesoterodine showed a good tolerability with reduced side effects [125].

Trospium chloride

Trospium chloride has a lower risk for drug-drug interactions because it is not metabolised by cytochrome P450 metabolising enzymes and, as it does not cross the blood-brain-barrier. Therefore, it is a viable alternative treatment for patients that cannot tolerate anticholinergics due to adverse effects or have a known mental decline. In several studies, trospium chloride was efficacious for OAB treatment, including urge incontinence [126].

Solifenacin

In a placebo- and tolterodine-controlled phase II dose-finding study, solifenacin demonstrated the best efficacy, quality of life, and tolerability at daily doses of 5 and 10 mg [127]. A prospective study over 12 weeks demonstrated a significant reduction of OAB symptoms and an improvement of QoL. Although gastrointestinal side effects were observed, solifenacin is generally well tolerated [128].

Darifenacin

The effect of the highly M_3 selective antagonist, darifenacin, was observed during a 12-week double-blind placebo-controlled, parallel-group study. Patients took darifenacin once daily whereby micturition frequency, bladder capacity, frequency and severity of urgency as well as number of incontinence episodes were significantly improved. However, there was no change in nocturia. The observed side effects were mild to moderate and neither show blurred vision nor cardiac or CNS related adverse events [129]. Darifenacin is suggested to be well tolerated showing no negative effects on the concentration, speed of response or short-term memory of patients in several studies. The low selectivity for M_1 receptors of the drug is a substantial advantage especially for elderly patients [130].

Calcium antagonist

Flavoxate HCl

Flavoxate (Urispas®) is a synthetic flavone derivative that has no anticholinergic side effects; therefore, it does not have an effect on muscarinic receptors. The relaxant effect of flavoxate on smooth muscle is induced by moderate L-type Ca^{2+} channel inhibition, local anaesthetic activity, and phosphodiesterase inhibition [131]. A double-blind crossover study demonstrated comparable efficacy for reducing urgency, frequency, and incontinence episode. Furthermore, flavoxate was associated with fewer and milder side effects [132].

β-Adrenoceptor agonists

β_3-Agonists were recently developed as a new approach to overactive bladder therapy. β_3-Agonists effect a bladder relaxation by acting on the contraction-inhibiting β_3-adrenoceptors.

Those drugs are expected to have fewer side effects than current anticholinergic drugs however an effect on tissues outside the bladder is not ruled out [133].

Mirabegron

In 2014, the first β_3-agonist agent, mirabegron (Betmiga®), was approved for the use in Switzerland and was indicated for OAB. A randomised, double-blind phase III study demonstrated a significant micturition frequency decrease over 24h and decreased incontinence events as well as improved QoL. During a 12-week treatment, doses of 50 mg or 100 mg once daily were well tolerated with dry mouth adverse events observed at comparable levels with placebo [134].

A randomised, double-blind phase II trial investigated the effect of the treatment combining mirabegron and solifenacin compared to the monotherapy with solifenacin 5 mg. The volume voided per micturition was increased in all combination groups. Also the micturition frequency and urgency episodes were improved in some of the combination groups. Overall, the combination groups showed the same safety profile like the monotherapy and placebo [135].

Tricyclic antidepressant

Imipramine

Imipramine (Tofranil®) is able to inhibit the detrusor muscle, which is assumed to result from a local anaesthetic effect on nerve terminals. Imipramine inhibits neuronal reuptake of serotonin and noradrenailne. The muscle relaxant effect is most probably exerted by the drug's influence on adrenoceptors and produces an increased outflow resistance in the bladder base and urethral smooth muscle [116,124]. Although imipramine should be carefully prescribed, the treatment of elderly patients with urinary incontinence and detrusor instability achieved either continence or increased bladder capacity and urethral closure pressure [136].

Oestrogens

Oestrogens are available as local and systemic therapies. A systematic review elucidated the effect of the oestrogen therapy on OAB in postmenopausal women. Locally applied oestrogens significantly improved micturition frequency, nocturia, urgency, number of incontinence episodes, first sensation to void, and bladder capacity. However, systemically administered oestrogens only significantly improved incontinence episodes and first sensation to void and nocturia worsened. In conclusion, locally administered oestrogens are more successful for OAB treatment than systemic oestrogens [137].

Onabotulinumtoxin A

When all traditional pharmacotherapies have failed or in the case of recurrent OAB, patients have the option of an onabotulinumtoxin A (BOTOX®) injection that is approved by Swissmedic for the indication of idiopathic overactive bladder since this year. Onabotulinumtoxin A is a neurotoxin that is produced by the anaerobic bacillus Clostridium botulinum. The preferred type A of the toxin shows a longer duration of action. Botulinum toxin prevents the release of Ach and neurotransmitters from presynaptic muscle cells, resulting in a reversible chemo-denervation of the detrusor muscle. The effect of ACh on postsynaptic muscle cells is inhibited and, therefore, the detrusor muscle does not contract [138]. Botulinumtoxin is injected directly into the detrusor muscle and showed in earlier publications a good efficacy for the treatment of neurogenic detrusor overactivity to reduce symptoms of urinary incontinence [139]. Different placebo-controlled studies also confirmed a good efficacy and tolerability for the treatment of idiopathic OAB. Its effect is noticeable a few days after injection and increases in the first 2-3 weeks. Normally, the therapy lasts for 9 to 11 months. A reinjection should not be performed before a time interval of 3 months. Botulinumtoxin acts as an antigen and therefore the precocious therapy can lead to a decreased effect [138].

2.3.6 *Bryophyllum pinnatum* - a new treatment option?

We have primarily focused on *B. pinnatum* and the possibility that it could be an alternative treatment for OAB patients.

A multicentre, randomised, double-blind placebo-controlled phase II study was performed as described in chapter 1.1.7.3. Women treated with *B. pinnatum* chewable tablets containing leaf press juice, reduced their micturition frequency/24h compared with placebo [63].

Furthermore, the effect of *B. pinnatu*m leaf press juice on porcine detrusor contractility was examined *in vitro*. Leaf press juice led to a relaxation of the carbachol pre-contracted muscle strips as well as inhibited electrically induced contractility [64]. In addition, the flavonoid fraction of *B. pinnatum* significantly inhibited porcine detrusor contractility in a dose-and time-dependent manner. The inhibitory effect of the flavonoid fraction was comparable with the anticholinergic drug oxybutynin (detailed information are provided in chapter 4.1 and 4.2) [65].

Additionally, we are currently examining the effect of *B. pinnatum* leaf press juice and the flavonoid fraction on human bladder detrusor muscle. Human bladders are obtained from patients after radical cystectomy. This *in vitro* study is approved by the Ethics Committee of Zurich (KEK-ZH-Nr. 2010-0021/4). We are including bladder samples from patients under the age of 70 years, who are affected by bladder cancer (< 50% of the bladder). Patients suffering from neurogenic bladders are not qualifying for the study. Prior to surgery, each

patient has to give oral and written informed consent. Until today, the bladders from nine patients were included, whereas it was not possible to isolate a bladder wall sample in two cases. The results to date confirm the observed inhibitory effect of the leaf press juice and the flavonoid fraction on electrically induced detrusor contractility. As compared with the effect on porcine detrusor contractility, a more potent effect was demonstrated as human detrusor muscles react more sensitive to *B. pinnatum* (data not shown).

These results indicate that *B. pinnatum* may be a promising therapy for OAB patients.

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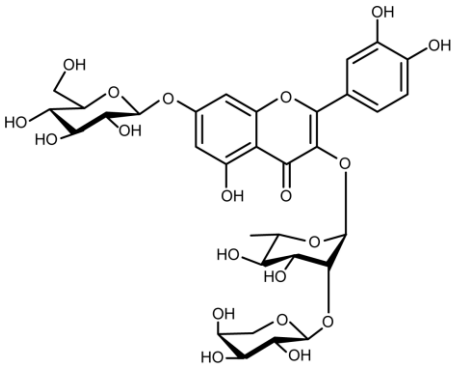
3 ANALYTICAL INVESTIGATIONS

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

1st publication

Fürer K, Raith M, Brenneisen R, Mennet M, Simões-Wüst AP, von Mandach U, Hamburger M, Potterat O. Planta Med 2013;79:1565-71.

This publication describes the isolation and identification of the constituents of *B. pinnatum*. After fractionating the MeOH leaf extract, two phenolic acid derivatives and nine flavonoid glycosides, including quercetin, kaempferol, myricetin, acacetin, and diosmetin glycosides, were identified using ^{1}H and 2D NMR spectroscopy. Quercetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside 7-O-*β*-D-glucopyranoside (**1**) and myricetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside (**2**) were identified as two new natural products. Bufadienolide reference substances were isolated from a CH_2Cl_2 fraction of another species, *B. daigremontianum*. Using these reference compounds, the presence of bersaldegenin-1-acetate, bryophyllin A, bersaldegenin-3-acetate, and bersaldegenin-1,3,5-orthoacetate was confirmed in *B. pinnatum*.



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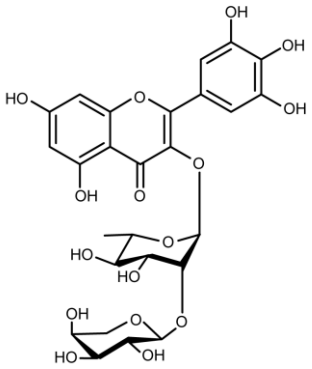

2

The preparation of the B. pinnatum MeOH extract, its fractionation (Sephadex LH-20 and Diaion HP-20 CC), purification of the constituents using semi-preparative and preparative HPLC, structure elucidation by NMR spectroscopy, isolation and identification of bufadienolides from B. daigremontianum, detection of bufadienolides in B. pinnatum, writing of the manuscript, and preparation of the figures and tables were my contributions to this publication.

Karin Fürer

Two New Flavonol Glycosides and a Metabolite Profile of *Bryophyllum pinnatum*, a Phytotherapeutic Used in Obstetrics and Gynaecology

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

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*.

Abbreviations

APCI:	atmospheric-pressure chemical ionisation
ESI:	electrospray ionisation
PDA:	photodiode array
CNS:	central nervous system
CC:	column chromatography

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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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 an 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].

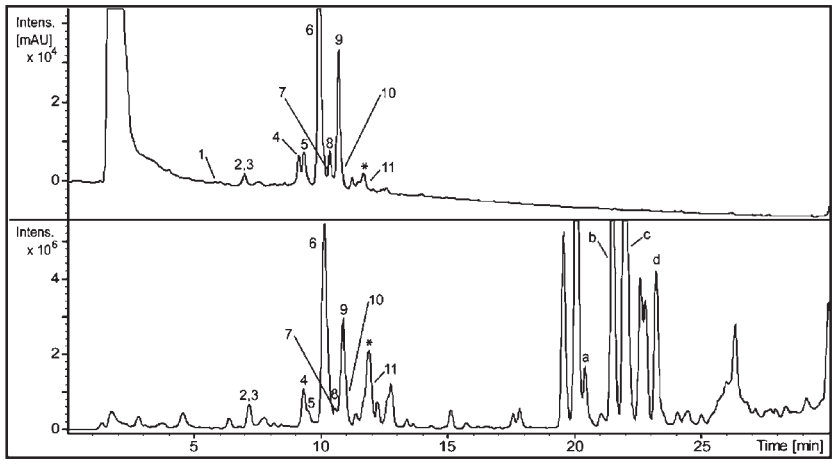

Fig. 1 HPLC-PDA-ESIMS of the MeOH extract of *B. pinnatum*. Top: UV trace (220–400 nm). Bottom: ESIMS base peak chromatogram (positive ion mode, *m/z* 200–1500). SunFire™ C_{18} column, A: 0.1% aqueous formic acid and B: MeCN, 5–100% B in A in 30 min, 0.5 mL/min.
Numbers refer to the isolated compounds **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 a large amount of malic acid, as revealed by ^{1}H NMR analysis.

With respect to pharmacological properties of *B. pinnatum,* antileishmanial [12], antiulcer [13], antibacterial [14, 15], antitumour 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 CNS-related activities [22]. For safety assessment and quality control of phytomedicines containing *B. pinnatum*, detailed information on their metabolite profile is required. There have been only a 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 was 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 semipreparative 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 $[2\,M + H]^+$) and **2** (*m/z* 651.2 $[2\,M - H]^-$, 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 $^3J_{HH}$-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 positions 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 deriva-

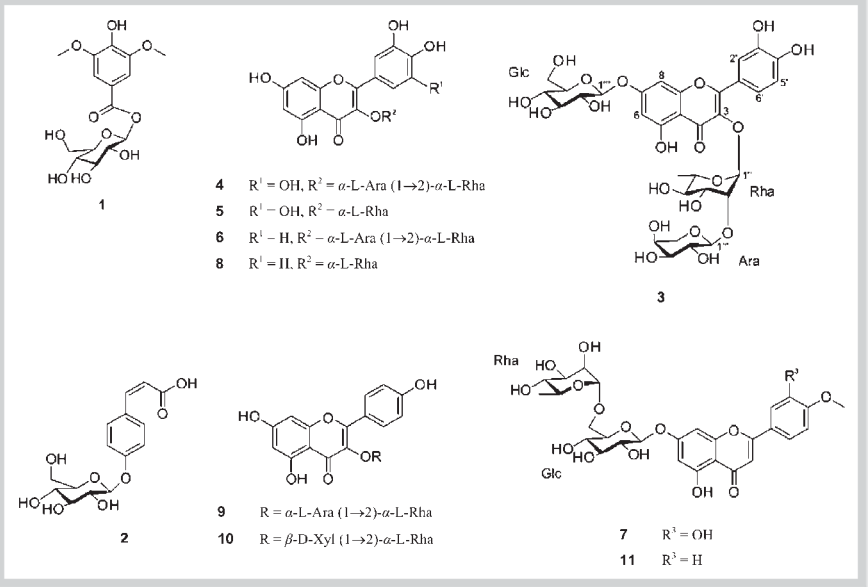

Fig. 2 Structures of compounds isolated from *B. pinnatum*.

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

tisation 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 → 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.

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

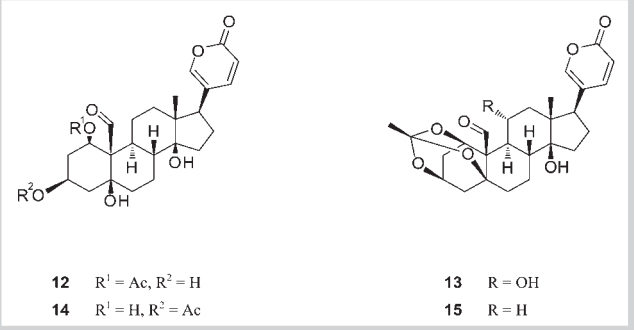

Fig. 3 Structures of bufadienolides isolated from *B. daigremontianum* and detected in *B. pinnatum*.

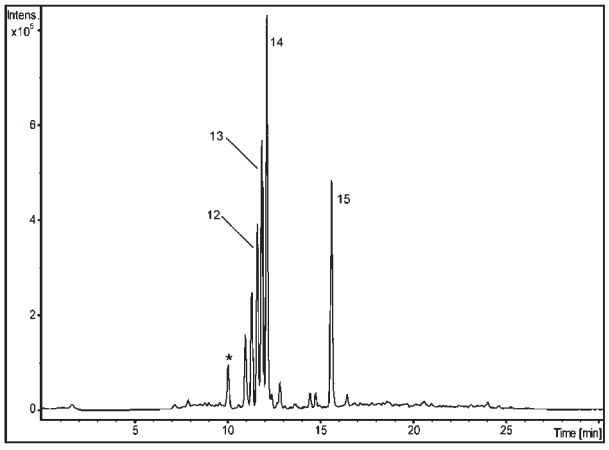

Fig. 4 Presence of bufadienolides in the CH_2Cl_2 soluble fraction of *B. pinnatum*: HPLC-APCIMS base peak chromatogram (positive ion mode, *m/z* 150–1500). SunFire™ C_{18} column, A: 0.1% aqueous formic acid and B: MeCN, 5–100% B in A in 30 min, 0.5 mL/min. Numbers refer to bufadienolides **12**-**15**. * 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.

Compound **4** differed from **6** only in the substitution of the B-ring. A signal corresponding to two protons appeared as a 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 → 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 semipreparative 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, ^1H and 2D NMR. ^1H 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 a 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 the 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 investiga-

tions 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 → 2)-α-L-rhamnopyranoside (**6**), confirming a previous report of its occurrence 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 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 the 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 $60_{F264\,s}$ (5–6 µm; Merck) were used for HPTLC with EtOAc/HCOOH/AcOH/H_2O (100 : 11 : 11 : 26) as the mobile phase. Detection was at 366 nm after spraying with Natural Product Reagent A. GC-MS analysis was performed using an HP 5890 Series II gas chromatograph equipped with an 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™ column (3.5 µm, 3 × 150 mm; Waters) equipped with a guard column (3 × 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. Semipreparative HPLC was carried out on an Agilent 1100 Series system connected to a PDA detector. Separations were performed on a Waters SunFire™ C_{18} column (5 µm, 10 × 150 mm) equipped with a precolumn (5 µm, 10 × 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™ C_{18} OBD™ column (5 µm, 30 × 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™ 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 pulverised 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 an additional 20 min. The extract was filtred 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 × 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 (Fr. 98–110, 0.17 g), B6 (Fr. 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 × 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 semipreparative 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); ^{1}H 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); ^{1}H 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 2 N HCl (1 mL) for 2 h. After cooling, the mixture was extracted with EtOAc (2 × 0.5 mL), and the aqueous phase freeze-dried. The sugars were redissolved in anhydrous pyridine, derivatised with L-cysteine methyl ester hydrochloride (200 µL, 60 °C, 1 h) and subsequently silylated with hexamethyldisilazane and chlorotrimethylsilane (Fluka) in pyridine (2 : 1 : 10; 300 µL; 60 °C, 30 min). GC-MS analysis was performed on a DB-225MS column (0.25 µm; 0.25 mm × 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 semipreparative 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 analysed 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 an employee of Weleda AG.

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Supporting Information

Two New Flavonol Glycosides and a Metabolite Profile of *Bryophyllum pinnatum*, a Phytotherapeutic Used in Obstetrics and Gynaecology

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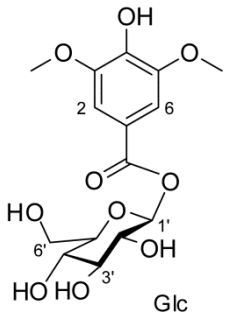
Table 1S ^{1}H and ^{13}C NMR data of compound **1** in DMSO-d_6

Syringic acid *β*-D-glucopyranosyl ester
$C_{15}H_{20}O_{10}$
M = 360.31 g/mol



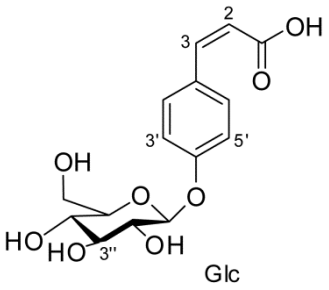
ESIMS (pos. ion mode): *m/z* 361.0 $[M + H]^+$, *m/z* 383.3 $[M + Na]^+$

position	δ_H (m, *J* in Hz)	δ_C [b)]	position[a)]	δ_H (m, *J* in Hz)	δ_C [b)]
Aglycone			*Glc*		
1	-	118.6	1'	5.54 (d, 7.7)	95.2
2	7.24 (s)	107.9	2'	3.30(m)	72.8
3	-	148.0	3'	3.27(m)	78.2
4	-	141.7	4'	3.20 (m)	70.1
5	-	148.0	5'	3.30 (m)	76.7
6	7.24 (s)	107.7	6'	3.66 (br d, 11.7), 3.49 (dd, 5.3, 12.0)	61.0
-COOH	-	164.9			
2x -OMe	3.79 (s)	56.6			

[a)]Glc = *β*-D-glucopyranosyl
[b)]^{13}C NMR shifts derived from HSQC and HMBC data

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

4'-O-*β*-D-glucopyranosyl-*cis*-p-coumaric acid
$C_{15}H_{18}O_8$
M = 326.30 g/mol



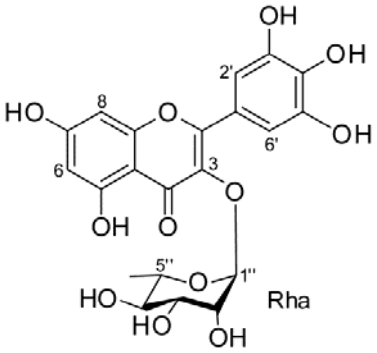
ESIMS (neg. ion mode): *m/z* 325.2 $[M - H]^-$, *m/z* 651.2 $[2M-H]^-$

position	δ_H (m, *J* in Hz)	δ_C [b)]	position[a)]	δ_H (m, *J* in Hz)	δ_C [b)]
Aglycone			*Glc*		
1	-	170.4	1''	4.88 (d, 4.9)	100.9
2	5.85 (d, 12.4)	123.6	2''	3.26 (dd, 6.7, 8.3)	73.8
3	6.53 (d, 12.9)	135.2	3''	3.30 (m)	77.0
1'	-	129.8	4''	3.20 (dd, 8.6, 8.7)	70.3
2'	7.67 (d, 7.5)	131.4	5''	3.34 (m)	77.5
3'	6.98 (d, 8.0)	115.9	6''	3.71 (d, 11.7), 3.50 (dd, 5.0, 11.3)	61.3
4'	-	157.8			
5'	6.98 (d, 8.0)	115.9			
6'	7.67 (d, 7.5)	131.4			

[a)] Glc = *β*-D-glucopyranosyl
[b)] ^{13}C NMR shifts derived from HSQC and HMBC data

Table 3S ^{1}H and ^{13}C NMR data of compound **5** in DMSO-d_6

Myricitrin (myricetin 3-O-*α*-L-rhamnoside)
$C_{21}H_{20}O_{12}$
M = 464.38 g/mol



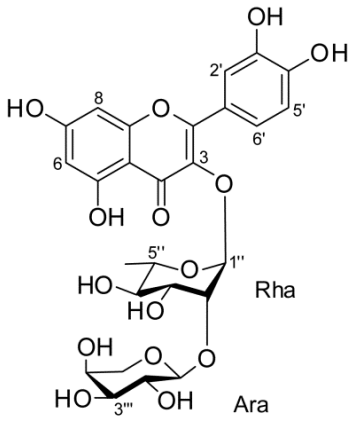
ESIMS (pos. ion mode): *m/z* 465.1 $[M + H]^+$, *m/z* 319.2 $[(M + H) - 146]^+$
ESIMS (neg. ion mode): *m/z* 463.7 $[M - H]^-$, *m/z* 927.2 $[2M - H]^-$

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	-	157.7	1''	5.23 (br s)	102.3
3	-	135.0	2''	4.00 (br s)	70.4
4	-	n.d.	3''	3.58 (m)	70.9
5	-	161.8	4''	3.18 (m)	71.9
6	6.21 (s)	99.0	5''	3.37 (m)	70.9
7	-	164.4	6''	0.86 (d, 6.0)	17.9
8	6.38 (s)	93.8			
9	-	156.8			
10	-	104.1			
1'	-	120.3			
2'	6.91 (s)	108.9			
3'	-	146.3			
4'	-	136.8			
5'	-	146.3			
6'	6.91 (s)	108.9			

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

Table 4S ^{1}H and ^{13}C NMR data of compound **6** in DMSO-d_6

Quercetin 3-O-*α*-L-arabinopyranosyl (1→2)-*α*-L-rhamnopyranoside
$C_{26}H_{28}O_{15}$
M = 580.50 g/mol



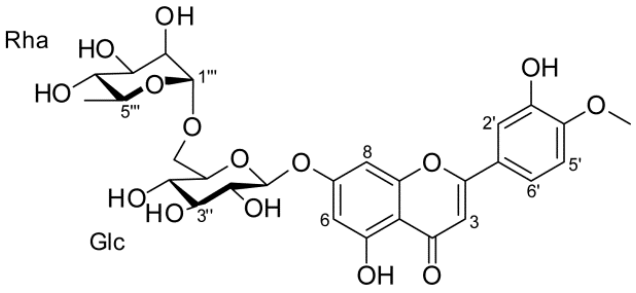
ESIMS (pos. ion mode): *m/z* 581.3 $[M+H]^+$, *m/z* 603.1 $[M+Na]^+$, *m/z* 449.2 $[(M + H) - 132]^+$, *m/z* 303.2 $[(M + H) - 132 - 146]^+$

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	-	157.1	1‘‘	5.34 (br s)	101.4
3	-	134.8	2‘‘	4.05 (br s)	80.8
4	-	n.d.	3‘‘	3.61 (m)	70.6
5	-	161.7	4‘‘	3.17 (dd, 9.4, 9.5)	72.3
6	6.21 (s)	99.0	5‘‘	3.61 (m)	70.6
7	-	164.6	6‘‘	0.92 (d, 6.0)	17.8
8	6.39 (s)	94.0			
9	-	156.2	*Ara*		
10	-	104.0	1‘‘‘	4.14 (d, 6.9)	106.6
1‘	-	121.0	2‘‘‘	3.36 (m)	71.7
2‘	7.35 (s)	116.2	3‘‘‘	3.33 (m)	72.8
3‘	-	145.9	4‘‘‘	3.58 (m)	68.3
4‘	-	149.3	5‘‘‘	3.50(m), 3.27(d, 11.7)	66.2
5‘	6.99 (d, 8.1)	116.2			
6‘	7.27 (d, 1.6, 8.3)	121.0			

[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

Table 5S ^{1}H and ^{13}C NMR data of compound **7** in DMSO-d_6

Diosmetin-7-O-*α*-L-rhamnopyranosyl (1→6)-*β*-D-glucopyranoside
$C_{28}H_{32}O_{15}$
M = 608.54 g/mol



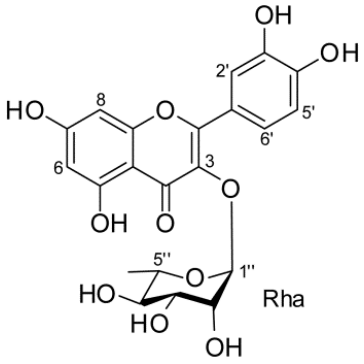
ESIMS (pos. ion mode): *m/z* 609.4 $[M+H]^+$

position	δ_H (m, *J* in Hz)	δ_C [b)]	position[a)]	δ_H (m, *J* in Hz)	δ_C [b)]
Aglycone			*Glc*		
2	-	164.5	1‘‘	5.08 (d, 6.8)	100.4
3	6.77 (s)	104.2	2‘‘	3.61 (m)	76.1
4	-	181.8	3‘‘	3.19 (m)	70.1
5	-	162.3	4‘‘	3.31 (m)	73.6
6	6.47(s)	100.1	5‘‘	3.35 (m)	76.8
7	-	163.2	6‘‘	3.88 (m),3.49 (m)	66.5
8	6.77(s)	95.2			
9	-	157.6	*Rha*		
10	-	105.8	1‘‘‘	4.59 (br s)	100.8
1‘	-	123.4	2‘‘‘	3.70 (m)	70.7
2‘	7.45 (s)	113.6	3‘‘‘	3.50 (m)	71.2
3‘	-	147.2	4‘‘‘	3.18 (m)	72.6
4‘	-	151.8	5‘‘‘	3.45 (m)	68.7
5‘	7.12 (d, 8.3)	112.8	6‘‘‘	1.09 (d, 5.7)	18.3
6‘	7.54 (d, 8.3)	119.3			
-OMe	3.89 (s)	56.2			

[a)] Rha = *α*-L-rhamnopyranosyl; Glc = *β*-D-glucopyranosyl
[b)] ^{13}C NMR shifts derived from HSQC and HMBC data

Table 6S ^{1}H and ^{13}C NMR data of compound **8** in DMSO-d_6

Quercitrin (quercetin 3-O-*α*-L-rhamnopyranoside)
$C_{21}H_{20}O_{11}$
M = 448.38 g/mol



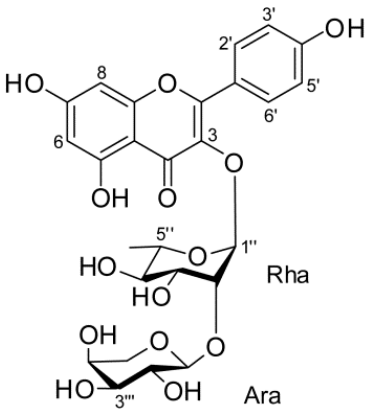
ESIMS (pos. ion mode): *m/z* 449.2 $[M+H]^+$, *m/z* 303.2 $[(M + H) - 146]^+$

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	-	157.8	1‘‘	5.28 (br s)	102.3
3	-	134.9	2‘‘	3.99 (br m)	70.5
4	-	n.d.	3‘‘	3.53 (dd, 2.8, 9.1)	70.9
5	-	161.6	4‘‘	3.17 (dd, 9.1, 9.3)	71.7
6	6.21 (s)	99.0	5‘‘	3.25 (dq, 6.2, 9.1)	70.9
7	-	164.5	6‘‘	0.84 (d, 6.0)	18.0
8	6.39 (s)	94.1			
9	-	156.5			
10	-	104.6			
1‘	-	121.2			
2‘	7.31 (s)	116.1			
3‘	-	145.7			
4‘	-	148.6			
5‘	6.88 (d, 8.3)	115.9			
6‘	7.26 (d, 7.0)	121.4			

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

Table 7S ^{1}H and ^{13}C NMR data of compound **9** in DMSO-d_6

Kaempferol 3-O-*α*-L-arabinopyranosyl (1→2)-*α*-L-rhamnopyranoside
$C_{26}H_{28}O_{14}$
M = 564.49 g/mol



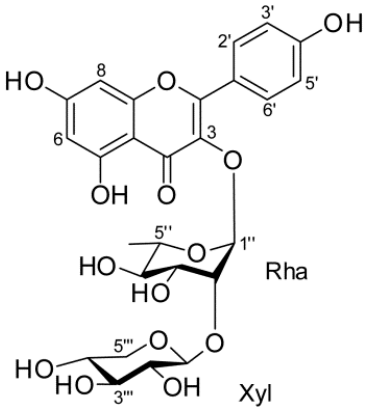
ESIMS (pos. ion mode): *m/z* 565.3 $[M+H]^+$, *m/z* 433.2 $[(M+H) - 132]^+$, *m/z* 287.2 $[(M + H) - 132 - 146]^+$

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	-	157.3	1‘‘	5.42 (br s)	101.2
3	-	134.7	2‘‘	4.03 (br m)	80.5
4	-	n.d.	3‘‘	3.57 (m)	70.8
5	-	161.7	4‘‘	3.15 (dd, 9.5, 9.5)	72.2
6	6.20(d, 1.8)	99.1	5‘‘	3.42 (dq, 6.3, 9.5)	70.8
7	-	165.1	6‘‘	0.89 (d, 6.2)	17.9
8	6.39(d, 1.8)	94.2			
9	-	156.4	*Ara*		
10	-	104.4	1‘‘‘	4.20 (d, 6.4)	106.5
1‘	-	120.8	2‘‘‘	3.37 (m)	71.6
2‘	7.76 (d, 8.6)	130.7	3‘‘‘	3.35 (m)	72.9
3‘	6.93 (d, 8.7)	116.4	4‘‘‘	3.57 (m)	68.0
4‘	-	160.1	5‘‘‘	3.56 (m), 3.30 (d, 11,8)	66.1
5‘	6.93 (d, 8.7)	116.4			
6‘	7.76 (d, 8.6)	130.4			

[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

Table 8S ^{1}H and ^{13}C NMR data of compound **10** in DMSO-d_6

Kaempferol 3-O-*β*-D-xylopyranosyl (1→2)-*α*-L-rhamnopyranoside
$C_{26}H_{28}O_{14}$
M = 564.49 g/mol



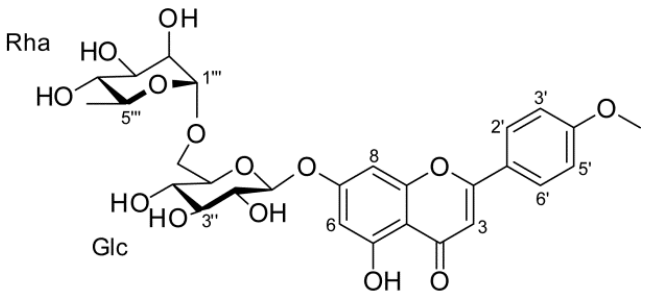
ESIMS (pos. ion mode): *m/z* 565.3 $[M+H]^+$, *m/z* 433.2 $[(M + H) - 132]^+$, *m/z* 287.2 $[(M + H) - 132-146]^+$

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	-	157.3	1‘‘	5.39 (br s)	101.2
3	-	134.7	2‘‘	4.01 (br m)	80.8
4	-	n.d.	3‘‘	3.57 (dd, 4.1, 9.3)	70.7
5	-	n.d.	4‘‘	3.14 (dd, 9.4, 9.4)	72.2
6	6.16 (s)	99.7	5‘‘	3.42 (dq, 6.4, 10.2)	70.7
7	-	n.d.	6‘‘	0.89 (d, 6.1)	17.8
8	6.35 (s)	94.4			
9	-	n.d.	*Xyl*		
10	-	104.1	1‘‘‘	4.21 (d, 7.5)	106.6
1‘	-	120.3	2‘‘‘	3.12 (dd, 8.8, 8.8)	76.6
2‘	7.75 (d, 8.5)	130.7	3‘‘‘	2.99 (dd, 8.3, 8.4)	74.1
3‘	6.93 (d, 8.5)	115.9	4‘‘‘	3.25 (dd, 4.7, 8.3)	69.8
4‘	-	160.6	5‘‘‘	3.57 (dd, 5.1, 11.7), 2.97 (br d, 11.3)	66.2
5‘	6.93 (d, 8.5)	115.9			
6‘	7.75 (d, 8.5)	130.7			

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

Table 9S ^{1}H and ^{13}C NMR data of compound **11** in DMSO-d_6

Acacetin 7-O-*α*-L-rhamnopyranosyl (1→6)-*β*-D-glucopyranoside
$C_{28}H_{32}O_{14}$
M= 592.54 g/mol



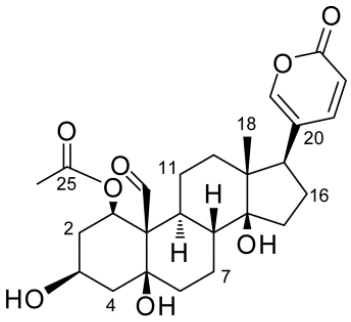
ESIMS (pos. ion mode): *m/z* 593.4 $[M+H]^+$
ESIMS (neg. ion mode): *m/z* 637.6 $[M + HCOO]^-$

position	δ_H(m, *J* in Hz)	δ_C [b)]	position [a)]	δ_H(m, *J* in Hz)	δ_C [b)]
Aglycone			*Glc*		
2	-	164.5	1''	5.06 (d, 7.1)	100.6
3	6.86 (s)	104.2	2''	3.61 (m)	76.2
4	-	182.5	3''	3.19 (m)	70.1
5	-	162.1	4''	3.31 (m)	73.6
6	6.45 (s)	100.2	5''	3.35 (m)	76.8
7	-	163.0	6''	3.89 (m), 3.48 (m)	66.5
8	6.77 (s)	95.2			
9	-	157.6	*Rha*		
10	-	106.0	1'''	4.59 (br s)	100.9
1'	-	123.1	2'''	3.70 (m)	70.8
2'	8.01 (d, 8.4)	128.5	3'''	3.50 (m)	71.3
3'	7.13 (d, 8.4)	115.1	4'''	3.18 (m)	72.5
4'	-	162.8	5'''	3.45 (m)	68.7
5'	7.13 (d, 8.4)	115.1	6'''	1.10 (d, 5.9)	18.2
6'	8.01 (d, 8.4)	128.5			
-OMe	3.85 (s)	55.9			

[a)] Rha = *α*-L-rhamnopyranosyl; Glc = *β*-D-glucopyranosyl
[b)] ^{13}C NMR shifts derived from HSQC and HMBC data

Table 10S ^{1}H and ^{13}C NMR data of compound **12** in DMSO-d_6

Bersaldegenin-1-acetate
$C_{26}H_{34}O_8$
M = 474.54 g/mol



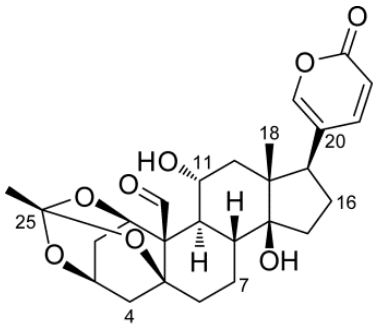
APCIMS (pos. ion mode): *m/z* 475.4 $[M+H]^+$

position	δ_H [b)](m, *J* in Hz)	δ_C [a),b)]
1	5.50 (br s)	70.7
2	1.95 (m), 1.89 (m)	30.9
3	4.11 (br s)	65.4
4	2.21 (br d, 14.4), 1.55 (m)	37.2
5	-	72.2
6	2.12 (m), 1.62(m)	36.2
7	1.46(m), 1.22 (m)	21.9
8	1.40 (d)	42.4
9	1.54 (br d, 10.9)	41.4
10	-	n.d.
11	n.d.	n.d.
12	1.38 (m), 1.28(m)	39.2
13	-	48.0
14	-	83.4
15	1.95 (m), 1.54(d, 10.9)	31.7
16	2.00 (m), 1.60 (m)	28.1
17	2.45 (dd, 6.6, 8.3)	50.2
18	0.51 (s)	17.0
19	10.00 (s)	206.6
20	-	122.9
21	7.49 (s)	149.4
22	7.87 (dd, 1.9, 9.8)	147.5
23	6.26 (d, 9.9)	114.4
24	-	161.7
25	-	169.9
26	1.85 (s)	21.5

[a)] ^{13}C NMR shifts derived from HSQC and HMBC data
[b)] n.d. not detected

Table 11S ^{1}H and ^{13}C NMR data of compound **13** in DMSO-d_6

Bryophyllin A
$C_{26}H_{32}O_8$
M = 472.53 g/mol



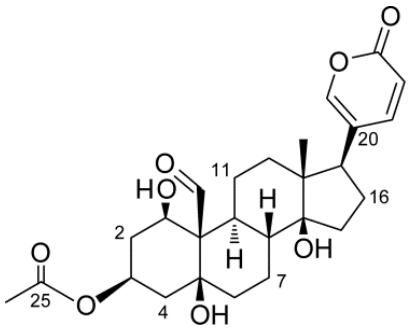
APCIMS (pos. ion mode): *m/z* 473.4 $[M+H]^+$

position	δ_H [b)](m, *J* in Hz)	δ_C [a),b)]
1	5.14 (d, 3.3)	72.9
2	2.15 (m),n.d.	27.9
3	4.25 (br s)	67.3
4	2.33 (d, 13.6),1.76(brd, 1.8, 13.3)	33.6
5	-	75.1
6	2.19 (ddd, 4.7, 13.5, 13.5), 1.50 (m)	33.3
7	2.05 (m), 1.27 (m)	22.3
8	1.50 (m)	41.3
9	1.64 (m)	46.9
10	-	n.d
11	3.79 (ddd, 4.4, 10.9, 10.9)	66.1
12	1.56 (m), 1.30 (m)	49.5
13	-	49.1
14	-	82.5
15	2.00 (m), 1.58 (m)	31.8
16	2.08 (m), 1.61 (m)	28.5
17	2.47 (dd, 5.5, 9.1)	50.2
18	0.55 (s)	17.7
19	10.10 (s)	207.8
20	-	122.4
21	7.52 (d, 1.6)	149.7
22	7.85 (dd,2.6, 9.8)	147.5
23	6.26 (d, 9.7)	114.4
24	-	161.7
25	-	110.0
26	1.21 (s)	26.3

[a)] ^{13}C NMR shifts derived from HSQC and HMBC data
[b)] n.d. not detected

Table 12S ^{1}H and ^{13}C NMR data of compound **14** in DMSO-d_6

Bersaldegenin-3-acetate
$C_{26}H_{34}O_8$
M = 474.54 g/mol



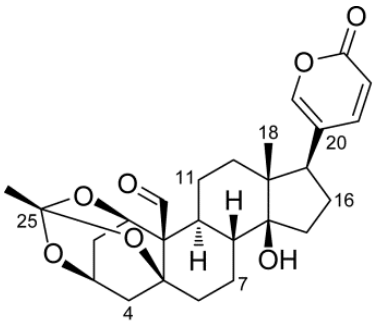
APCIMS (pos. ion mode): *m/z* 475.5 $[M+H]^+$

position	δ_H(m, *J* in Hz)	δ_C [a)]
1	4.43 (br s)	68.8
2	2.01(m), 1.94(m)	31.1
3	5.06(br s)	68.7
4	1.59(m), 2.31(m)	36.1
5	-	73.2
6	2.15 (ddd, 4.3, 13.5, 13.7), 1.51(m)	36.1
7	2.02(m), 1.14(m)	24.3
8	1.41(m)	42.2
9	1.56(m)	41.1
10	-	56.0
11	1.42(m), 1.35(m)	21.4
12	1.34(m), 1.24(m)	39.6
13	-	48.0
14	-	83.1
15	1.93(m), 1.51(m)	31.9
16	2.02(m), 1.58(m)	28.5
17	2.44(dd, 9.4, 5.4)	50.4
18	0.53 (s)	17.1
19	10.10 (s)	208.4
20	-	122.9
21	7.49 (d, 1.7)	149.4
22	7.88 (dd, 2.5, 9.8)	147.6
23	6.26 (d, 9.7)	114.4
24	-	161.7
25	-	170.1
26	1.95 (s)	21.6

a)^{13}C NMR shifts derived from HSQC and HMBC data

Table 13S ^{1}H and ^{13}C NMR data of compound **15** in $CDCl_3$

Bersaldegenin-1,3,5-orthoacetate
$C_{26}H_{32}O_7$
M = 456.53 g/mol



APCIMS (pos. ion mode): *m/z* 457.3 $[M+H]^+$

position	δ_H(m, *J* in Hz)	δ_C [a)]
1	4.51 (br s)	70.6
2	2.41 (m), 1.59(m)	27.2
3	4.26 (br s)	66.6
4	2.14 (br s), 1.91 (m)	33.5
5	-	74.4
6	2.24 (ddd, 4.1, 13.5, 13.5), 1.57 (m)	32.6
7	2.05 (m), 1.32 (m)	22.2
8	1.56 (m)	42.5
9	1.44 (m)	41.1
10	-	52.6
11	1.52 (m), 1.29 (m)	20.7
12	1.44 (m), 1.19 (m)	40.2
13	-	48.7
14	-	84.0
15	1.84 (m), 1.63(m)	31.7
16	2.10 (m), 1.69 (m)	28.3
17	2.38 (m)	50.8
18	0.59 (s)	16.1
19	10.10 (s)	206.7
20	-	122.3
21	7.15 (s)	148.5
22	7.68 (dd, 1.5, 9.9)	146.3
23	6.16 (d, 6.2)	115.1
24	-	161.7
25	-	110.8
26	1.32 (s)	25.7

[a)] ^{13}C NMR shifts derived from HSQC and HMBC data

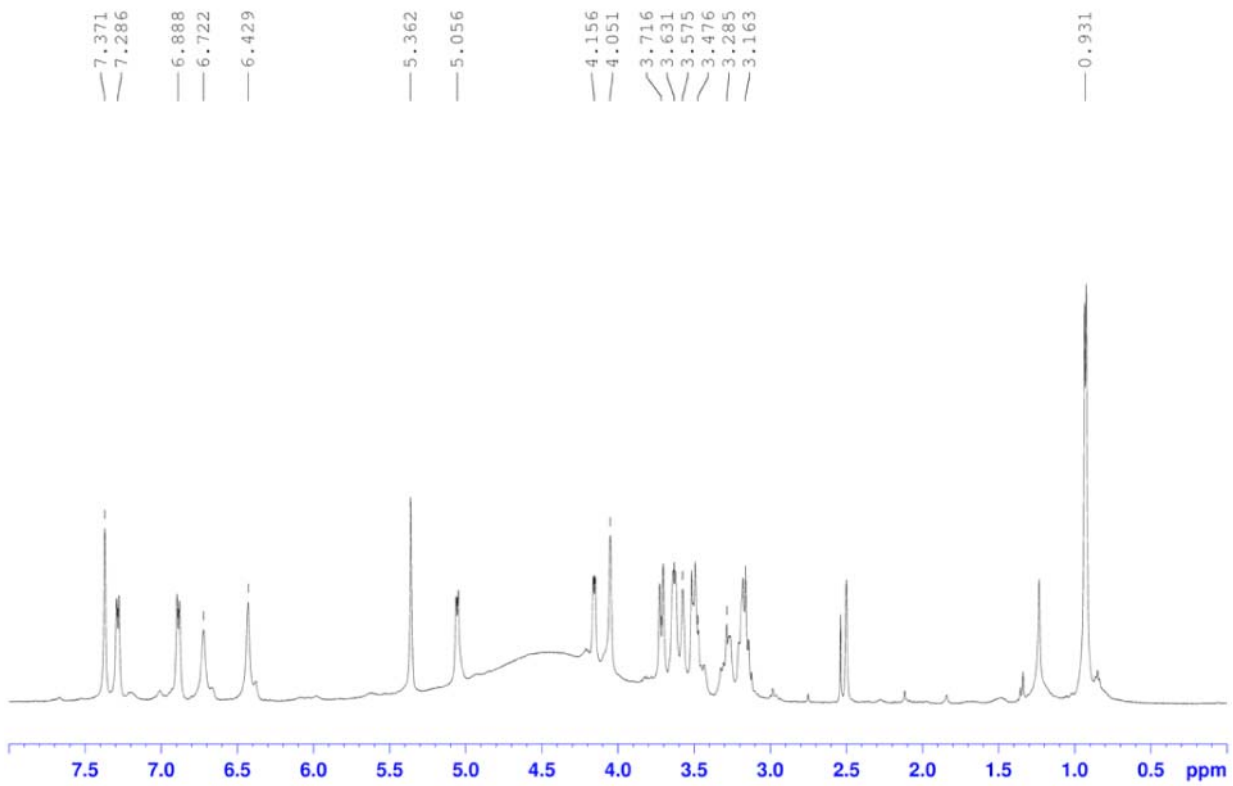


Fig 1S ^{1}H NMR spectrum of compound **3** in DMSO-d_6

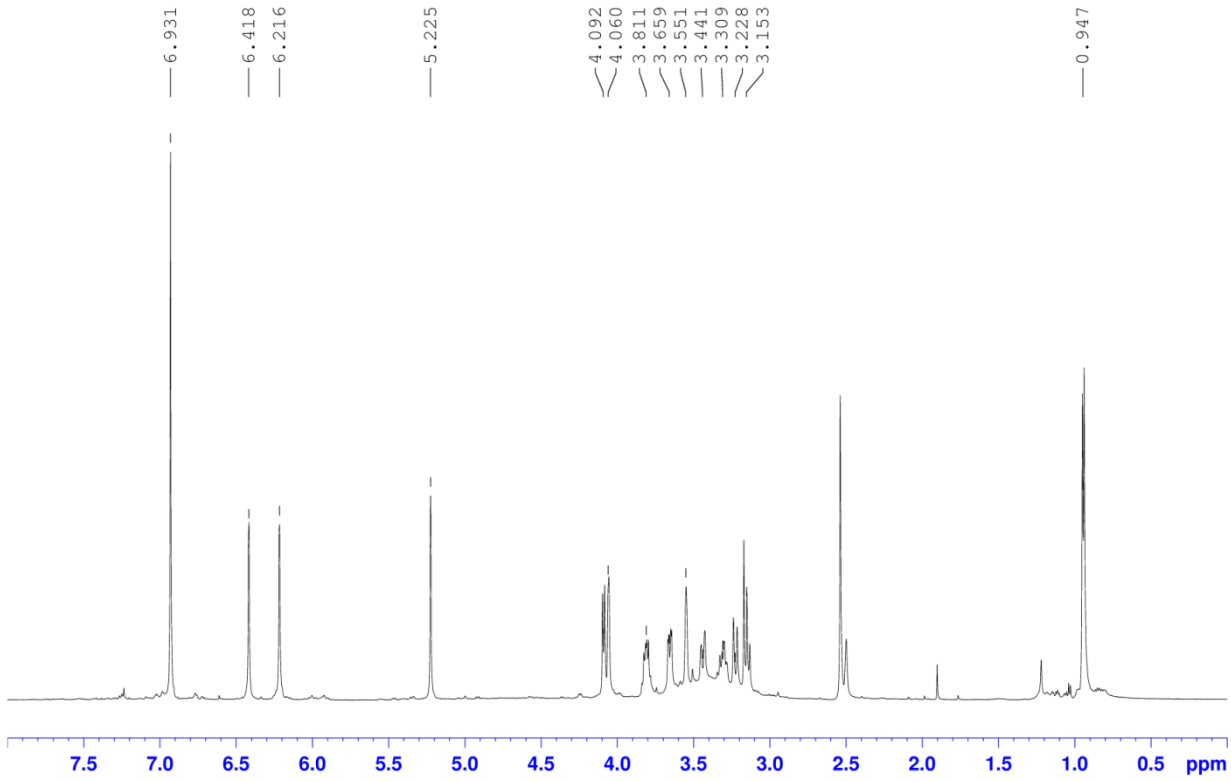


Fig 2S ^{1}H NMR spectrum of compound **4** in DMSO-d_6

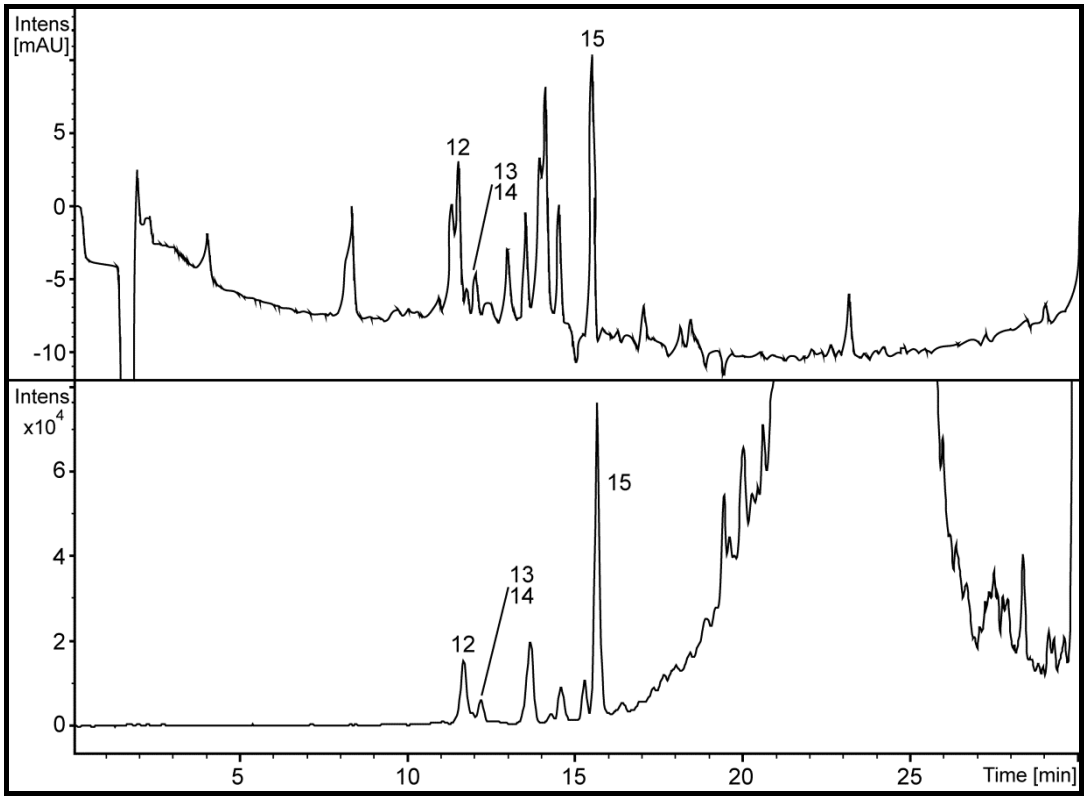


Fig 3S HPLC-PDA-APCIMS of the CH_2Cl_2soluble fraction of *B. daigremontianum*. Top: UV 298 nm trace. Bottom: HPLC-APCIMS base peak chromatogram (positive ion mode, *m/z* 150 - 1500). SunFire^{TM} C_{18} column, A: 0.1% aqueous formic acid and B: MeCN, 5-100% B in A in 30 min, 0.5 mL/min. Numbers refer to bufadienolides **12-15**.

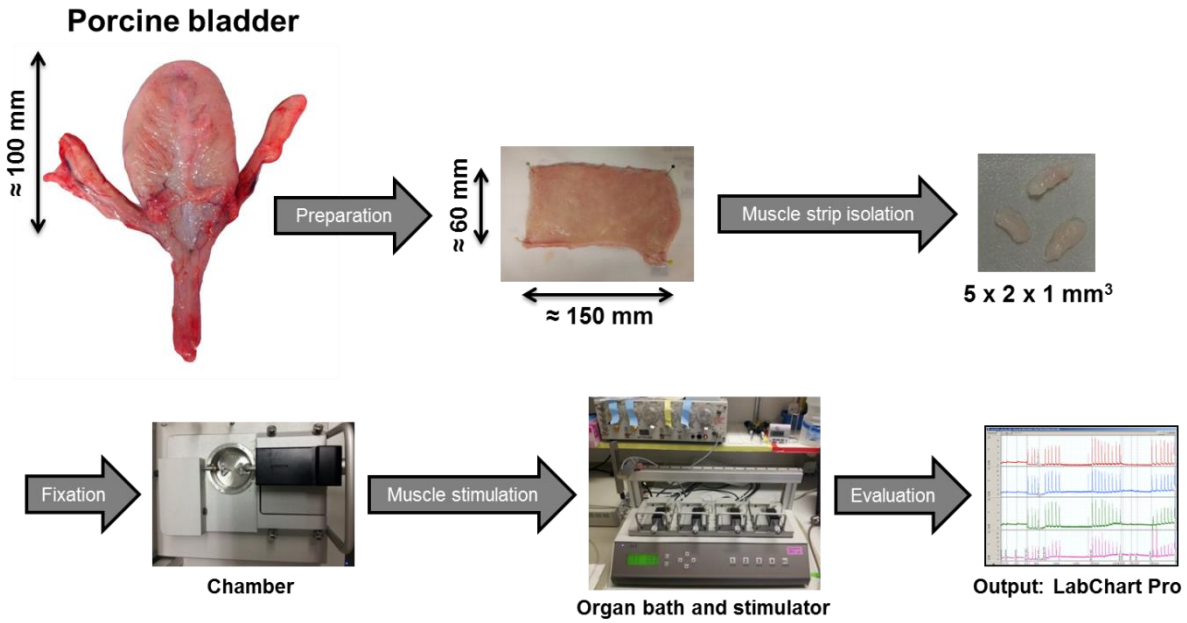
4 PHARMACOLOGICAL EXPERIMENTS

4.1 *Bryophyllum pinnatum* inhibits detrusor contractility in porcine bladder strips - A pharmacological study towards a new treatment option of overactive bladder

2nd publication

Schuler V, Suter K, Fürer K, Eberli D, Horst M, Betschart C, Brenneisen R, Hamburger M, Mennet M, Schnelle M, Simões-Wüst AP, von Mandach U. Phytomedicine 2012;19:947-51.

The effect of the *B. pinnatum* leaf press juice on the porcine detrusor contractility was demonstrated *in vitro*. The leaf press juice (10%) produced a maximum relaxation of 18.7% on carbachol pre-contracted detrusor contractility and a maximum inhibition of 74.6% in electrically stimulated muscle strips compared with the control at 10% and 5% concentrations, respectively.



The initial in vitro experiments with porcine bladders and supporting the master thesis were my contributions to this publication.

Karin Fürer

Phytomedicine 19 (2012) 947–951

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Bryophyllum pinnatum inhibits detrusor contractility in porcine bladder strips—A pharmacological study towards a new treatment option of overactive bladder

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ABSTRACT

Aims: A broad spectrum of synthetic agents is available for the treatment of overactive bladder. Anticholinergic drugs show a poor compliance due to side effects. There is an increasing use of plant extracts in medicine. We have therefore investigated the inhibitory effects of leaf press juice from Bryophyllum pinnatum (Lam.) Oken (Kalanchoe pinnata L.) on bladder strips and compared the effects to that of oxybutynin.

Methods: Strips of porcine detrusor were prepared in Krebs solution and contractility was measured in a myograph system chamber aired with O_2/CO_2 at 37 °C. To induce contractions, electrical field stimulation (32 Hz, 40 V) was used for the inhibitory effect measurements, and carbachol (50 μM) for the relaxant effect measurements. Recordings were obtained in the absence and presence of increasing concentrations of Bryophyllum pinnatum leaf press juice (BPJ, 0.1–10%), and oxybutynin (10^{-7}–10^{-3} M) as a reference substance.

Results: In inhibition experiments, BPJ as well as oxybutynin inhibited electrically induced contractions of porcine detrusor. BPJ at concentrations of 5% inhibited the contraction compared to a time matched control significantly by 74.6 ± 10.2% ($p < 0.001$). BPJ as well as oxybutynin relaxed carbachol pre-contracted porcine detrusor strips. The maximum relaxant effect of BPJ compared to a time matched control was 18.7 ± 3.7 ($p < 0.05$) at a concentration of 10% BPJ.

Conclusions: Our investigations show that BPJ inhibits contractions induced by electrical field stimulation and relaxes carbachol-induced contractions. However, the effect was lower than that of the reference substance oxybutynin. It is important to continue *in vitro* experiments as well as clinical studies with BPJ that might offer a new treatment option for patients with OAB.

Introduction

Lower urinary tract dysfunction causing urgency with or without urge incontinence, usually accompanied by frequency and nocturia, was defined by the International Continence Society (ICS) in 2002 as the overactive bladder (OAB) syndrome (Abrams et al. 2002). This new term, which replaces older terms such as unstable bladder/detrusor instability and detrusor hyperreflexia, is a symptomatic diagnosis. The fundamental cause of OAB remains to be discovered and there is some evidence that myogenic (Brading 1997), aberrant neurogenic activity (DeGroat 1997) and cerebral alterations (Griffiths et al. 2005) as well as atypical or latent bladder infections (Kavia et al. 2005) are involved in its pathogenesis. Involuntary detrusor contractions that are of clinical importance and demonstrable during urodynamic fill cystometry can be simulated in an *in vitro* model of bladder muscle strips.

OAB syndrome affects a considerable part of the population: Prevalence of OAB is at least as high as the rates of many other

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chronic diseases, including asthma, coronary-artery disease and peptic-ulcer disease (Chapple et al. 2008). Depending on the definition for OAB which were used in different epidemiologic studies, prevalence values range from 11.8% (Irwin et al. 2006) to 16.6% (Milsom et al. 2001). Only Irwin et al. based their multinational, population based, cross-sectional survey on the current ICS definitions. All of the mentioned studies found similar prevalence rates of OAB in men and women, and an increase in prevalence with older age.

OAB affects many aspects of daily life, like domestic behaviour, social interactions, sexuality, work life, physical activities and overall psychological well-being. Nevertheless, Milsom et al. revealed that only a small proportion of affected individuals currently receive treatment: Only 60% of the interviewed individuals had spoken to a doctor about their disorders, and among these, 73% remained without medication.

The management of OAB includes a broad spectrum of treatments, such as behaviour modification, pharmacotherapy, pelvic floor muscle training, electrostimulation and surgery. Antimuscarinics, such as oxybutynin, tolterodine, darifenacin, solifenacin, fesoterodin, and trospium chloride are the only oral drug class to demonstrate a positive benefit-to-risk ratio. Anticholinergics remain the first line treatment for OAB (Andersson 2011). However, the lack of selectivity of antimuscarinics to the muscarinergic receptors of the bladder muscle and urothelial cells leads to systemic anticholinergic side effects, such as dry mouth, tachycardia, cognitive problems, constipation and accommodation paralysis (i.e., blurred vision). These side effects limit the clinical use of this drug class, especially in the elderly (Andersson 2011). Long-term compliance in an observational study was low, with less than 20% of subjects continuing the medication after 6 months (Kelleher et al. 1997). In a recent prospective tolerability and efficacy study, the compliance of the participants was much higher with 61% after 12 weeks (Herschorn et al. 2010).

For these reasons, interest in novel drug development for a more effective and well-tolerated treatment of OAB has increased in recent years. As OAB is a multilevel disease involving bladder, neuronal and cerebral aspects, a new drug is expected to modulate all levels in a favourable manner.

Complementary medicine systems like phytotherapy or anthroposophic medicine are appreciated by a growing number of patients and physicians, due to good tolerability and efficacy in various diseases. Phytomedicines are quite popular in some European countries where they may be prescribed by medical doctor, and fully or partially reimbursed by the health care system.

Bryophyllum pinnatum (Lam.) Oken (*B. pinnatum*, Crassulaceae) is a perennial plant originating from Madagascar. The plant is growing widely in tropical Africa, tropical America, India, China and Australia, and is known by a multitude of synonyms and common names like *Kalanchoe pinnata* Lam., *B. calycinum* Salisb., life plant, air plant, love plant, Canterbury bells, Cathedral bells, etc. Its uses in folk medicine have been as diverse as its names and included applications as an antimicrobial, antifungal, antiprotozoal, antiulcer, antiinflammatory, analgesic, sedative, muscle relaxant, antihypertensive and antiallergic (Kamboj and Saluja 2009). In Europe, the use of remedies prepared from the species *B. pinnatum* is limited almost exclusively to anthroposophic medicine where it was introduced by Rudolf Steiner in 1921 for treatment of "hystery" (Hamre et al. 2006). Since 1970, *B. pinnatum* is used in anthroposophically oriented hospitals as routine treatment of preterm labour (Plangger et al. 2006).

Some phytochemical studies from *B. pinnatum* have been conducted which led to the identification of bufadienolides (Yamagishi et al. 1989), flavonoids, flavonoid glycosides (Muzitano et al. 2006), and several phenolics and organic acids (Muzitano et al. 2006) from *B. pinnatum*. However, no pharmacological studies with extracts nor isolated compounds have been conducted prior to our investigations (Wächter et al. 2011).

An *in vitro* study showed that *B. pinnatum* reduced the contractility of myometrium muscle strips in both spontaneous and stimulated contractions (Gwehenberger et al. 2004). We recently demonstrated a relaxant effect of *B. pinnatum* leaf press juice (BPJ) and some chromatographic fractions on myometrium strips (Gwehenberger et al. 2004).

Encouraged by these findings, we tested the effect of BPJ on detrusor contractility in an *in vitro* porcine bladder model.

Materials and methods

Plant material

B. pinnatum plants were provided by Weleda Brazil. A voucher specimen number ZSS 29717 is deposited at the Zurich Succulent Plant Collection, Switzerland. The plants (leaves) were harvested in Brazil (03/15/2010) by Mr. Moaci Copani, Weleda Brazil, in the morning before flowering. Thereafter the fresh plant material was placed in a refrigerated box and immediately sent by airplane to Weleda Arlesheim, Switzerland. The plants were kept refrigerated and processed within 3 days of arrival by mechanical pressing in a roller mill to obtain BPJ (Weleda 03/23/2010). The procedure corresponded to the production process for the active ingredient of Weleda. Bryophyllum 50% tablets (Weleda AG, Arlesheim). The juice was stored in aliquots of 1.5 ml at −80 °C.

Chemicals and solutions

Carbamyolinecholine chloride (carbachol) and oxybutynin chloride were purchased from Sigma–Aldrich (Schnelldorf, Germany).

The Krebs solution contained (in mmol/l): 119 NaCl (AppliChem,) 15.0 $NaHCO_3$ (Merck, Darmstadt, Germany), 4.6 KCl (Haenseler AG, Herisau, Switzerland), 1.5 $CaCl_2$ (Merck, Darmstadt, Germany), 11.0 Glucose (Sigma–Aldrich, Steinheim, Germany), 1.2 $MgCl_2 \times 6\ H_2O$ (Merck, Darmstadt, Germany), 1.2 $NaH_2PO_4 \times 1\ H_2O$ (Merck, Darmstadt, Germany). The solution was adjusted to a pH of 7.4 and stored at 4 °C.

Stock solutions of carbachol (10^{-1} M) and oxybutynin (10^{-2} M) were stored at −80 °C, dilutions with Krebs solution were freshly prepared for each experiment.

124 mM KCl-Krebs solution contained (in mmol/l): 124 KCl (Haenseler AG, Herisau, Switzerland), 1.5 $CaCl_2$ (Merck, Darmstadt, Germany), 1.2 $MgCl_2 \times 6\ H_2O$ (Merck, Darmstadt, Germany), 15.0 $NaHCO_3$ (Merck, Darmstadt, Germany), 1.2 $NaH_2PO_4 \times 1\ H_2O$ (Merck, Darmstadt, Germany), 11.0 Glucose (Sigma–Aldrich, Steinheim, Germany). The solution was adjusted to a pH 7.4 and stored at 4 °C. Ecotainer® Aqua (B. Braun, Sempach, Switzerland) was used as the solvent for all solutions.

Porcine urinary bladder

Due to similar density and pharmacological characteristics of the muscarinic receptors in porcine and human bladder tissue (Sellers and McKay 2007), the consistency of electrical properties of detrusor smooth muscle from the porcine and human urinary bladder (Hashitani and Brading 2003), and good accessibility of porcine bladders, we decided to carry out the contractility experiments on porcine detrusor strips. Empty porcine urinary bladders were obtained from a local slaughterhouse where they were immediately immersed in cool Krebs solution for the transport. The mucosal layers were removed, and detrusor strips (2 × 1 × 5 mm) were cut in a longitudinal direction from the bladder wall. Isometric tension recordings were performed within 7 h.

Functional experiments

Porcine detrusor strips were mounted in a 6 ml tissue bath chamber (610M, Muscle Strip Myograph System, DMT, Denmark, supplied by ADInstruments GmbH, Spechbach, Germany) filled with Krebs solution, serated with 95% O_2 and 5% CO_2 at 37 °C. The tissue was fixed between two clamps. Contractile responses were recorded and digitized using AD Instruments PowerLab force transducer model 4/30 in connection with LabChartPro 7.0 software (ADInstruments, Sydney, Australia). An initial equilibration period of 0.5 g for a minimum of 30 min was allowed before starting the experiments.

Inhibitory effect measurements: Electrical field stimulation (EFS) was applied through a Grass S48 electrical stimulator (Astro-Med inc., W. Warwick, RI, USA) and two electrodes placed on each side of the strips. Contractions were induced by squared twin pulses of 40 V, 32 Hz, with a duration of 2 ms, given for 5 s with an interval of 3 min. The first stimulation after the equilibration period was performed to get the maximum amplitude of the contraction; thereby the mean of 3 similar peaks was used. Subsequently BPJ (0.1, 0.5, 1, 2.5, 5 and 10%) was added cumulatively followed by electrical stimulation after each BPJ application. The incubation time for each BPJ concentration was 5 min and the next higher concentration was added after three similar amplitudes. One strip per run without addition of any substance was used as control. Oxybutynin (10^{-5} to 10^{-3} M) was used as a reference substance.

Relaxant effect measurements: The strips were stimulated with 50 μM carbachol to obtain a constant level of tension. Then BPJ was added with cumulative concentrations (0.1, 0.5, 1, 2.5, 5 and 10%). In the following washout phase, Krebs solution was changed 4 times until the strips achieved baseline tension. Carbachol 50 μM was added again to check the contractility of the strips after the treatment with BPJ. Oxybutynin (10^{-7} to 10^{-5} M) was used as a reference substance. One strip of every run was used as a time-matched control without addition of any substance.

Data analysis and statistics

In the EFS inhibition experiments, the mean of 3 amplitudes before addition of BPJ or oxybutynin was defined as 100% contraction. Values after addition of BPJ, oxybutynin or the control, respectively, were expressed as the percentage of the 100% value; for each concentration the mean of 3 amplitudes was used. In relaxation experiments, the difference between the mean of amplitudes before the addition of carbachol (=baseline) and the mean immediately after the addition of carbachol was defined as 100% contraction. The relaxant effect after addition of each concentration of BPJ or oxybutynin was expressed as a percentage of the 100% value. Results of the experiments are expressed as mean ± standard error of the mean (SEM). Statistical significance of differences between BPJ or oxybutynin and the time-matched controls were determined using an unpaired Student's two-tailed *t*-test (Excel, Microsoft Office 2007) with a probability level of $p < 0.05$ being considered significant.

Results

A total of 32 bladders were used for the experiments.

Inhibition of electrically induced contractions

BPJ ($n = 7$) reduced the muscle tension to 87.1 ± 5.4%, 53.4 ± 8.7%, 32.5 ± 10.2% and 33.2 ± 16.8% at concentrations of 1, 2.5, 5 and 10% BPJ, respectively. Compared to the time matched control group ($n = 12$), BPJ showed a significant inhibition at 1, 2.5, 5 and 10% BPJ ($p < 0.05$, $p < 0.01$, $p < 0.001$); the maximum inhibitory effect was 74.6 ± 10.2% at the concentration of 5% ($p < 0.001$) (Fig. 1A).

Oxybutynin ($n = 6$) reduced the muscle tension to 53.1 ± 9.4%, 28.9 ± 6.3%, 19.4 ± 5.1%, 11.0 ± 4.0%, 5.9 ± 2.4% and 4.0 ± 1.4% at concentrations between 10^{-5} and 10^{-3} M, respectively. Compared to the time matched control group ($n = 12$) it showed a significant inhibition at 10^{-5} to 10^{-3} M ($p < 0.0001$); the maximum inhibitory effect was 96.0 ± 1.4% at a concentration of 10^{-3} M ($p < 0.0001$) (Fig. 1B).

Relaxation of carbachol induced contractions

BPJ ($n = 9$) and the anticholinergic reference substance oxybutynin ($n = 6$) produced a concentration-dependent relaxation of the carbachol pre-contracted detrusor strips. BPJ was able to reduce the tension to 53.6 ± 3.1% and 48.3 ± 3.7%. Compared to the time matched control group ($n = 11$), BPJ showed a significant relaxation at 5 and 10% ($p < 0.05$); the maximum relaxant effect was 18.7 ± 3.7 at 10% BPJ (Fig. 2A).

Oxybutynin was able to reduce the tension to 52.4 ± 5.6%, 30.3 ± 5.3%, 21.6 ± 3.9%, 15.1 ± 3.0%, and 10.4 ± 3.2% at concentrations of $10^{-6.5}$ M, 10^{-6} M, $10^{-5.75}$ M, $10^{-5.5}$ M and 10^{-5} M, respectively. Compared to the time matched control ($n = 11$) oxybutynin showed a significant relaxation ($p < 0.001$) at all concentrations except 10^{-7} M; it led to a maximum relaxant effect of 56.7 ± 3.2% at 10^{-5} M (Fig. 2B).

Discussion

Despite the high prevalence and the considerable impact on quality of life, pharmacological treatment options for patients suffering from OAB are still limited. Herbal drugs are generally considered to be as effective as synthetic products. The identification of a nature-derived drug with a good activity and tolerability would be a relief for both patients suffering from OAB and their attending physician.

B. pinnatum is widely used in traditional medicine in different parts of the world (Kamboj and Saluja 2009). Its muscle relaxant activity to human myometrium *in vitro* and *in vivo* has been shown before (Plangger et al. 2006; Wächter et al. 2011; Gwehenberger et al. 2004). Therefore, we investigated the inhibitory and relaxant effect of BPJ on the contractions of isolated porcine detrusor muscle strips induced by electrical and chemical stimulation.

This experiment shows that BPJ in concentrations of 1–10% leads to an inhibition of electrically induced contractions. The inhibition was more pronounced for oxybutynin than for BPJ at all concentrations investigated (0.01–1 μM *vs.* 1–10%, respectively). However, it is difficult to compare the results of a press juice with a chemically defined substance since the compounds responsible for the activity of BPJ and their concentration are not completely characterised yet. Both substances led to a concentration-dependent relaxation of carbachol-induced contractions. However, the inhibitory effect on the contractility of porcine detrusor muscle was more distinguished than the relaxant. One reason for the divergent results in these two experiments could be that the signalling pathways leading to contraction and relaxation of the detrusor smooth muscle differ. The stimulation of muscarinic receptors on the detrusor by acetylcholine, which is physiologically released from activated cholinergic nerves, is the main mechanism of bladder contraction (Chapple et al. 2008). This stimulation is known to be competitively inhibited by oxybutynin, our comparative study drug for relaxation and inhibition measurements. The M_3-receptor subtype predominantly mediates bladder contraction despite the large majority of the M_2-receptor subtype expressed in the bladder mucosa (Chapple et al. 2008). The role of the M_2-receptor subtype

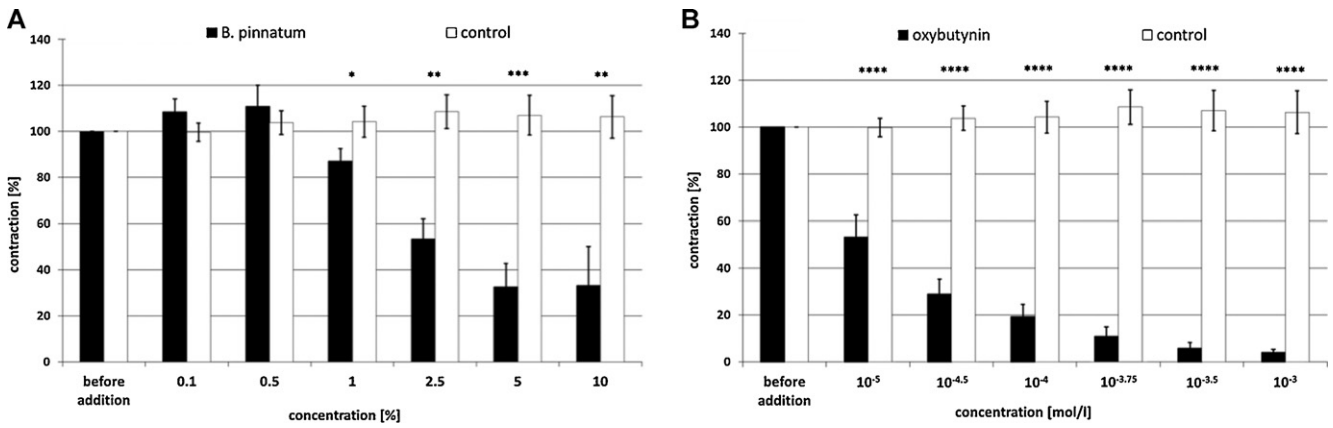

Fig. 1. Inhibitory effect of *B. pinnatum* leaf press juice (A) and oxybutynin (B), respectively, on electrically induced contractions of porcine detrusor muscle. 100% of the contraction was defined as mean of the 3 amplitudes before addition of the drug in increasing concentrations. The strips were stimulated after each addition of the drug. Mean values ± SEM of the treated group and of the time-matched control group are shown. Appropriate values were compared to those of the control group by the unpaired Student's two tailed *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

is not yet clarified. The M_2-receptor may play an indirect role in mediating bladder contractions by enhancing the contractile response to M_3-activation (Chapple 2000). However, an explanation for our results is that the strong inhibitory effect of BPJ may be triggered by the inhibition of M_3-receptors, whereas the relaxant effect is mediated by the stimulation of the M_2-receptor subtype. Since the expression of muscarinic receptors and the electrical properties of detrusor smooth muscle from the porcine and human urinary bladder are very similar (Sellers and McKay 2007), *B. pinnatum* may have a comparable effect on human detrusor.

Oxybutynin was shown to effectively block carbachol-induced contractions in numerous investigations. Its affinity for M_1- and M_3-receptors exceeds that of M_2-subtypes (Andersson 2011). Besides cholinergic control of detrusor contractions, other mechanisms of actions have been discussed. They include, among others, the inhibition of the influx of extracellular calcium into detrusor muscle cells by L-type Ca^{2+}-channel blockers or K^+-channel openers (Sacco et al. 2008).

Taking into account the favourable side effect profile of BPJ in previous studies (Plangger et al. 2006), a muscarinic independent or balanced pathway might be suggested. Since BPJ contains a multitude of different compounds which are poorly studied, we cannot conclude on their potential affinity to muscarinic or adrenergic receptors, Ca^{2+} or K^+ channels or other targets. We recently showed that *B. pinnatum* inhibits the oxytocin-induced increase of intracellular calcium concentration in human myometrial cells in a dose-dependent, but extracellular Ca^{2+}-independent, manner. At the same time *B. pinnatum* delays (but not fully prevents) the depolarization-induced increase in intracellular Ca^{2+} in cells known to express L-type Ca^{2+}-channels (Simões-Wüst et al. 2010). Whether a comparable delay can explain the now observed inhibitory effect on the contraction of detrusor smooth muscle cells deserves further investigation. In addition, radioligand displacing studies would possibly offer a suitable method to investigate the receptor - or ion channel-binding properties of BPJ constituents in detrusor smooth muscle cells.

Furthermore, we recently showed that a flavonoid-containing fraction from BPJ was pharmacologically active and produced relaxation of the myometrium (Gwehenberger et al. 2004).

Other plants have been previously investigated for their *in vitro* inhibitory properties on detrusor contractility, such as Ylang Ylang (*Cananga odorata*) (Kim et al. 2003), St. John's Wort (*Hypericum perforatum*) (Capasso et al. 2004), European gold rod (*Solidago virgaurea*) (Borchert et al. 2004) and sweet sumach bark (*Rhus aromatica*) (Borchert et al. 2004). However, none of these herbal drugs have been further developed towards a novel treatment option for OAB, and the need for effective and well tolerated phytopharmaceuticals remains. Since *B. pinnatum* has been shown to effectively inhibit uterine contractions *in vitro* (Gwehenberger et al. 2004), and to produce few and only moderate side effects *in vivo* (Plangger et al.

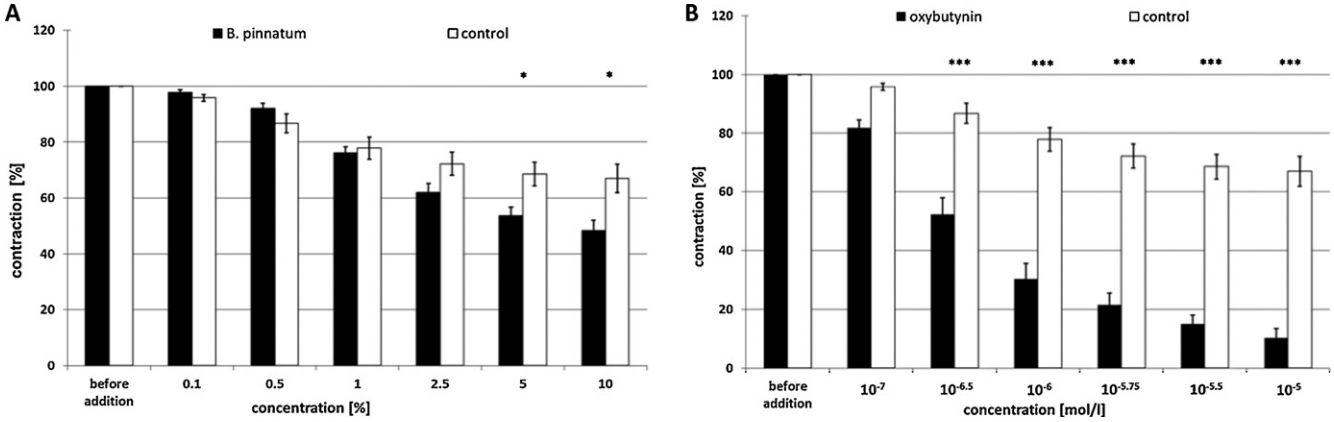

Fig. 2. Relaxant effect of *B. pinnatum* leaf press juice (A) and oxybutynin (B), respectively on carbachol-induced contractions of porcine detrusor muscle. Tension of 100% represents the difference between the level before and after addition of 50 μM carbachol. The drug in each concentration was cumulatively added to the strips. Mean values ± SEM of the treated group and of the time-matched control group are shown. Appropriate values were compared to those of the control group by the unpaired Student's two tailed *t*-test. $^{*}p < 0.05$ and $^{***}p < 0.001$.

2006) this plant appears highly promising as a future treatment option for OAB disorders. Identification of the pharmacologically active compounds in the extract, and an investigation of their mechanism of action are planned as next steps in the development of *Bryophyllum pinnatum* towards a potential new treatment of overactive bladder.

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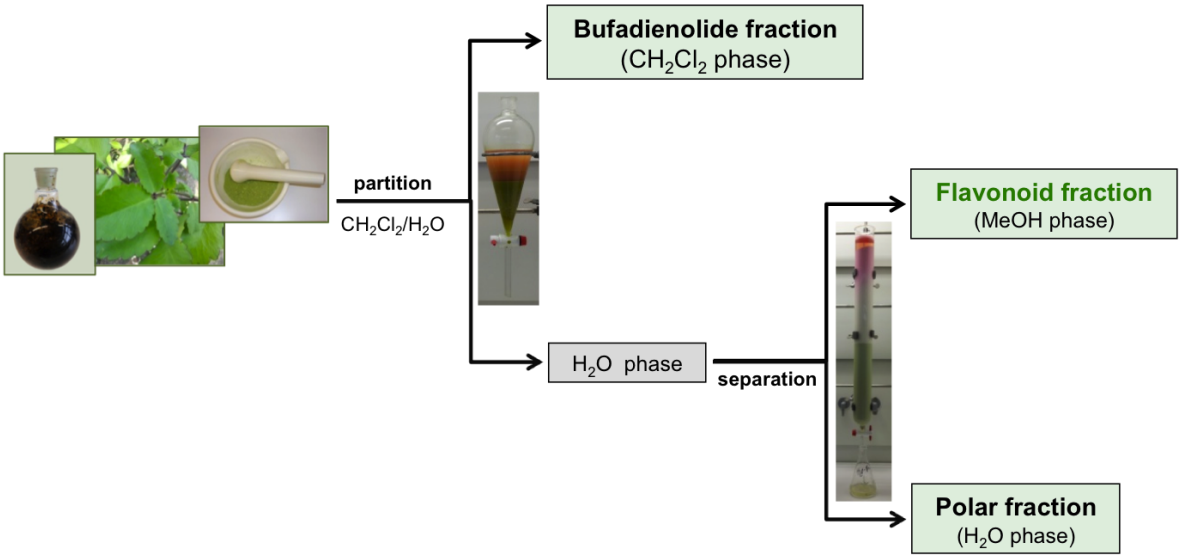
4.2 Inhibition of porcine detrusor contractility by the flavonoid fraction of *Bryophyllum pinnatum* - a potential phytotherapeutic drug for the treatment of the overactive bladder syndrome

3rd publication

Fürer K, Eberli D, Betschart C, Brenneisen R, De Mieri M, Hamburger M, Mennet-von Eiff M, Potterat O, Schnelle M, Simões-Wüst AP, von Mandach U. Phytomedicine 2014;submitted.

This publication demonstrated the effect of the leaf press juice as well as of the flavonoid, bufadienolide, and polar fractions of *B. pinnatum* on porcine detrusor contractility *in vitro*. The leaf press juice was able to inhibit electrically stimulated detrusor concentrations in an organ bath. The flavonoid fraction (1 mg/mL) produced a 78.7% maximum inhibition of detrusor contractility. Muscle strips treated with the bufadienolide fraction demonstrated no inhibitory effects on detrusor contractility. The polar fraction demonstrated an unexpected inhibitory effect; however, this effect may be explained by a lower pH in the organ bath chamber caused by the presence of L-malic acid. Flavonoids are hypothesized to be responsible for the inhibitory effect on porcine detrusor muscle contractility.



The preparation of the flavonoid, bufadienolide, and polar fractions (liquid-liquid extraction and Diaion HP-20 CC), identification of the fractions constituents, the method development, in vitro experiment performance, the pH measurements, writing of the manuscript, and preparation of the figures (except for Fig. S3, S4) and tables were my contributions to this publication.

Karin Fürer

Inhibition of porcine detrusor contractility by the flavonoid fraction of *Bryophyllum pinnatum* – a potential phytotherapeutic drug for the treatment of the overactive bladder syndrome

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Keywords

Kalanchoe pinnata, flavonoids, overactive bladder, detrusor muscle, muscle relaxation, muscle contractility

Abbreviations

APCI: Atmospheric-pressure chemical ionization

BPJ: *Bryophyllum pinnatum* leaf press juice

$[Ca^{2+}]_i$: Intracellular free calcium concentration

CC: Column chromatography

EFS: Electrical field stimulation

ESI: Electrospray ionization

HPLC: High-performance liquid chromatography

OAB: Overactive bladder syndrome

PDA: Photodiode array

ABSTRACT

Aims: To determine if the phytotherapeutic agent, *Bryophyllum pinnatum*, could serve as an alternative drug for the overactive bladder syndrome, and to characterise the fraction responsible for the inhibition of detrusor contractility.

Methods: Fractions were prepared from the MeOH extract of *B. pinnatum* and further analysed by HPLC-PDA-MS. Detrusor muscle strips were prepared from porcine bladders and the electrically induced muscle contractility measured by organ bath. The effect of *B. pinnatum* leaf press juice (2.5 - 10%), a flavonoid fraction (0.1 - 1 mg/mL), and a bufadienolide fraction (0.1 - 40 µg/mL) on detrusor contractility was assessed and compared with controls (polar fraction (0.5 - 5 mg/mL) and oxybutynin (10^{-8} - 10^{-6} M)).

Results: The press juice, at a concentration of 10% led to a reduction of detrusor contractility. Bladder strips treated with the flavonoid fraction showed a significant reduction of the contractility to 21.3 ± 5.2% (1 mg/mL) while the bufadienolide fraction had no inhibitory effect in the investigated concentrations. The polar fraction showed a reduction of the contractility in a pH-dependent fashion. At 10^{-6} M concentration oxybutynin reduced the detrusor contractility to 21.9 ± 4.7%. *Conclusions:* The flavonoid fraction of *Bryophyllum pinnatum* reduces the porcine detrusor contractility in a dose- and time-dependent manner. Fractions from *B. pinnatum* may be a new pharmacological approach for the treatment of OAB.

INTRODUCTION

Overactive bladder syndrome (OAB) is a symptomatic diagnosis that was defined by the International Continence Society (ICS) as urgency, with or without urge incontinence, usually with frequency and nocturia, after exclusion of a bladder infection or other obvious pathology (Haylen et al., 2010).

In 2008, the worldwide prevalence was 10.7%, and is expected to rise to 20.1% in 2018 (Irwin et al, 2011). OAB often impacts negatively the quality of life especially in case of incontinence. However, many affected individuals do not seek help and therefore do not receive a treatment. Patients suffering from a neurological disease (e.g. multiple sclerosis, Parkinson's disease, stroke) have a higher incidence. Changes in lifestyle, bladder training, and pelvic floor muscle exercises are the first step of conservative treatments (Hashim and Abrams, 2004).

Antimuscarinic drugs such as oxybutynin are used as first-line pharmacotherapy with a proven clinical benefit. However, many patients suffer from anticholinergic side effects, lack of efficacy, or unfulfilled treatment expectations. Up to 20% of the patients fail to respond adequately, or their expectations were too high (Veenboer and Bosch, 2014).

As a new therapeutic approach β_3-adrenoceptor agonists have been investigated. Mirabegron was recently introduced as a treatment of OAB. A multicenter randomized double-blind study showed good efficacy and tolerability compared to placebo (Khullar et al., 2013; Chapple et al., 2013).

If conservative treatment fails, surgical interventions like botulinum toxin injection, sacral nerve stimulation, or detrusor myectomy are recommended as further options (Toozs-Hobson, 2010).

Given the drawbacks of the above-mentioned approaches, alternative pharmacological treatment options would be of value for patients suffering from OAB. Previous research indicated that *Bryophyllum pinnatum* could be such a new therapeutic approach.

Bryophyllum pinnatum (Lam.) Oken (syn. *Kalanchoe pinnata* Pers., *Bryophyllum calycinum* Salisb., Crassulaceae) is a succulent perennial plant native to Madagascar. The leaves have been used in traditional, native, and anthroposophical medicine (Kamboj and Saluja, 2009; Daems et al., 1982). Since 1970, *B. pinnatum* is used as a tocolytic agent to prevent premature labour (Hassauer et al., 1985). Leaf press juice and a flavonoid fraction of *B. pinnatum* were shown to induce myometrial relaxation *in vitro* (Wächter et al., 2011).

Similar effects could be demonstrated on bladder detrusor muscle where *B. pinnatum* leaf press juice had an inhibitory effect on electrically or carbachol-induced porcine detrusor muscle contractility (Schuler et al., 2012). In a pilot study with 22 postmenopausal women suffering from OAB, a positive effect of leaf press juice on the frequency of micturition compared to placebo could be shown after 8 weeks of treatment (Betschart et al., 2013).

Based on these findings, we further investigated the effect of *B. pinnatum* on porcine detrusor contractility. Herbal extracts contain a multitude of different phytochemicals. *B. pinnatum* is known to contain various flavonoids, bufadienolides, alkaloids, triterpenes, phytosterols, fatty acids, minerals, and vitamins (Kamboj and Saluja, 2009). For scientifically founded phytomedicines an identification

of the pharmacologically active constituents is important. In that perspective a phytochemical profiling of *B. pinnatum* was performed. We isolated and identified two phenolic acid derivatives and nine flavonoid glycosides, and identified four bufadienolides with the aid of reference substances isolated from the taxonomically related *B. daigremontianum* (Fürer et al., 2013). The latter species is known to contain higher amounts of bufadienolides, which have been shown to exert positive inotropic and CNS related activites (Wagner et al., 1986).

The aim of this study was to identify the active compounds/fractions of *B. pinnatum* responsible for the inhibitory effect on porcine detrusor muscle contractility. For that purpose a flavonoid, a bufadienolide fraction, and a control fraction containing highly polar compounds of *B. pinnatum* leaf extract were prepared and tested on porcine detrusor muscle strips.

MATERIAL AND METHODS

Plant material

B. pinnatum leaf press juice (BPJ): Leaves were harvested in March 2010 from plants cultivated in Brazil by Weleda Brazil. The leaves were immediately frozen and sent by air mail to Weleda Arlesheim, Switzerland. The BPJ (03-23-2010) was produced within 3 days of arrival by mechanical pressing of leaves. BPJ was aliquoted into 1.5 mL samples and stored at -80°C. A voucher specimen (ZSS 29717) has been deposited at the Zurich Succulent Plant Collection.

B. pinnatum leaves: Leaves were harvested in August 2010 from plants cultivated in Schwäbisch Gmünd by Weleda Schwäbisch Gmünd, Germany. The leaves were kept frozen until processing. A voucher specimen (ZSS 29715) has been deposited at the Zurich Succulent Plant Collection.

Instruments and chemicals

For extraction and column chromatography (CC) technical grade solvents (Scharlau, Germany) were used after distillation. HPLC grade solvents were used for HPLC. HPLC grade water was obtained by an EASY-pure II (Barnstead) water purification system.

HPLC-PDA-MS analyses were performed using an Agilent 1100 Series HPLC system coupled to a Bruker Esquire 3000 plus mass spectrometer equipped with ESI or APCI source. MS data were recorded in positive ion mode. Separations were achieved on a SunFire^{TM} C_{18} column (3.5 µm, 3 x 150 mm, Waters) equipped with a guard column (3 x 10 mm). 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 with a flow rate of 0.5 mL/min was applied.

Preparative fractionation of extract on Diaion HP-20 (250 µm, Supelco) was achieved in a glass column (70 x 700 mm).

^{1}H NMR spectra were recorded at 23°C in DMSO-d_6 (Armar Chemicals, Switzerland) on a Bruker Avance III^{TM} 500 MHz NMR spectrometer equipped with a 5-mm BBI probe. Data were processed with Topspin 3.0 software (Bruker). For quantification of L-malic acid, 1,3,5-trimethoxybenzene (>99,

Sigma-Aldrich) was used as internal standard. Quantitative ^{1}H NMR was carried out using the pulse program zg (Bruker) with a recycle delay of 20 sec (>5xT1). The sample was prepared and measured in triplicate.

Reference substances used for compound identification were previously isolated from the MeOH extract of *B. pinnatum* and from the CH_2Cl_2-soluble fraction of the MeOH extract of *B. daigremontianum* (Fürer et al., 2013).

Extraction

Frozen leaves of *B. pinnatum* were lyophilised, and pulverised in a mortar. The powdered leaves (1064.7 g) were extracted with MeOH (10 L). The suspension was stirred 2h at r.t. and subsequently sonicated for additional 20 min. The extract was filtered, and evaporated under reduced pressure to yield the MeOH extract (262.4 g).

Preparation of fractions from the MeOH extract

A portion (111.9 g) of the MeOH extract was partitioned between CH_2Cl_2 and H_2O, and each phase was washed twice. The CH_2Cl_2-soluble phase was evaporated to yield the bufadienolide-containing fraction (10.8 g).

The aqueous phase was fractionated by CC with Diaion HP-20 resin. Elution with H_2O gave 56.5 g of a highly polar fraction, and elution with MeOH afforded 12.0 g of a flavonoid fraction.

Identification of the constituents of the fractions

The flavonoid, bufadienolide, and polar fractions were analysed by HPLC-PDA-MS. ESI and APCI were used as ionization modes. The samples were dissolved in DMSO at a concentration of 1 mg/mL, and aliquots of 20 µL were injected. Peak identification was achieved by chromatographic comparison with reference compounds, and by analysis of PDA and MS data (Fürer et al., 2013).

A phenolic acid derivative and nine flavonoid glycosides were identified in the flavonoid fraction by ESIMS analysis (Fig. 1): syringic acid *β*-D-glucopyranosyl ester (1), quercetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside 7-O-*β*-D-glucopyranoside (2), myricetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside (3), myricitrin (4), quercetin 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside (5), diosmine (6), quercitrin (7), kaempferol 3-O-*α*-L-arabinopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside (8), kaempferol 3-O-*β*-D-xylopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside (9), and acacetin 7-O-*α*-L-rhamnopyranosyl-(1 → 6)-*β*-D-glucopyranoside (10) (Fig. 2).

APCIMS analysis revealed four bufadienolides, which were unambiguously identified in the bufadienolide fraction (detailed results are provided as supporting information): bersaldegenin-1-acetate (11), bryophyllin A (12), bersaldegenin-3-acetate (13), and bersaldegenin-1,3,5-orthoacetate (14) (Fig. 3).

Constituents of the polar fraction could not be identified by ESIMS and APCIMS. Therefore, ^{1}H NMR analysis of the polar fraction was performed to identify its constituents. Based on the ^{1}H NMR spectrum, the polar fraction contains mainly L-malic acid as well as different sugars (detailed results are provided as supporting information).

Porcine bladder muscle strips

For *in vitro* contractility experiments the Krebs solution (pH 7.4) containing (in mmol/L) 119.0 NaCl (AppliChem, Darmstadt, Germany), 15.0 $NaHCO_3$ (Sigma-Aldrich, Steinheim, Germany), 11.0 Glucose (Sigma-Aldrich, Steinheim, Germany), 4.6 KCl (Zurich canton pharmacy, Zurich, Switzerland), 1.5 $CaCl_2$ (Dr. Bender & Dr. Hobein AG, Zurich, Switzerland) 1.2 $MgCl_2$ x 6 H_2O (AppliChem, Darmstadt, Germany), 1.2 NaH_2PO_4 x H_2O (Merck, Darmstadt, Germany) was used (Badawi et al., 2006).

Oxybutynin chloride (Fig. 4), L-malic acid, D-malic acid and succinic acid were purchased from Sigma-Aldrich.

Porcine urinary bladders were obtained from the slaughterhouse in Zurich. They were from 5 months old female pigs weighing around 100 kg. Immediately after slaughtering, bladders were kept in Krebs solution. All experiments were done within 9 hours after removal of bladders.

Bladders were emptied before cutting off the dome and trigone. The urothelium was carefully removed from the detrusor. After, the serosa was removed and longitudinal muscle strips (5 x 2 x 1 mm^3, 25 - 40 mg) were prepared. Eight muscle strips were deposited in Krebs solution (on ice) until use.

Organ bath measurements

The experiments were performed with a DMT 610M Multi Chamber Myograph connected to an ADInstruments PowerLab 4/30 signal transducer and to a Grass S48 Electrical field stimulator. In the organ bath chamber (Krebs solution aired with 5% CO_2 and 95% O_2, 6 mL, 37°C) the strips were fixed between two jaws, which were connected either to a force transducer or a micropositioner.

The electrical field stimulation (EFS) was performed with squared twin pulses with duration of 2 ms and an interval of 3 min. The muscle strips were stimulated for 5 seconds with a constant voltage and frequency (40 V, 32 Hz) and the contraction force recorded.

After attaching the muscle strips, an equilibrium phase with 10 mN was applied. The stretching of the muscle strips was repeated after 5 and 10 min. Krebs solution was exchanged every 10 min. After 30 min of equilibration, the baseline contraction force of each strip was set to zero. Before application of test solutions, the muscle strips were stimulated 7 times each 3 min by EFS to determine the maximum contraction of each strip.

BPJ, fractions, oxybutynin, or control solution (Krebs solution or DMSO (0.5%)) were added in the corresponding concentration to the organ bath chamber. The following concentrations were tested:

BPJ (2.5, 5, 10% (v/v)), flavonoid fraction (0.1, 0.33, 0.4, 0.5, 0.64, 0.8, 1 mg/mL in DMSO), bufadienolide fraction (0.1, 0.5, 2, 5, 15, 30, 40 µg/mL in DMSO), polar fraction (0.5, 1, 2, 3, 4, 5 mg/mL in Krebs solution), and oxybutynin (10^{-8}, 10^{-7}, 10^{-6} M in Krebs solution). After an incubation time of 5 min, the muscle strips were electrically stimulated 24 or 28 times, in intervals of 3 min. This was followed by a washout phase, whereby, for 30 min the Krebs solution was renewed every 6 min. After that, the muscle strips were stimulated 9 times to check the muscle vitality.

A total of 35 porcine bladders were used to treat n = 7 muscle strips per concentration for all test samples.

Data analysis and statistics

The contractility (amplitude of the peak) of the muscle strips was recorded by LabChart 7 Pro (ADinstruments) and analysed with the peak analysis module. The mean of 6 - 7 muscle contractions before treatment was set as the maximum contraction (100%). The contractility after treatment was expressed as a percentage of the maximum contraction of the muscle strips. The data are presented either as mean or as mean ± standard error of the mean (SEM). For the statistical evaluation a two-way ANOVA was performed by GraphPad Prism 5 comparing the results of the test solution to the control. A significance level $p<0.05$ was considered statistically significant.

RESULTS

To investigate the contribution of the different constituents of *B. pinnatum* on porcine detrusor contractility, three district fractions were prepared from the MeOH extract. A fraction rich in bufadienolides was obtained by liquid/liquid partition with dichloromethane, while the remaining aqueous phase was subjected to CC with HP-20 to afford a polyphenol-rich fraction consisting mainly of flavonoid glycosides, and a highly polar fraction containing sugars and L-malic acid.

HPLC-ESI-MS analysis of the flavonoid fraction revealed the presence of a phenolic acid derivative and nine flavonoid glycosides. No further compounds and, in particular, no bufadienolides were detected by HPLC-APCI-MS analysis.

Five bufadienolides were identified in the bufadienolide fraction by APCI-MS. In addition, a flavonoid glycoside, and lysophosphatidylcholine derivatives were detected by ESI-MS analysis of the bufadienolide fraction.

The compounds contained in the polar fraction could not be identified by HPLC-PDA-MS analysis. ^{1}H NMR analysis of the polar fraction revealed the presence of large amounts of L-malic acid, and a mixture of different sugars. With the aid of quantitative NMR, the content of L-malic acid in this fraction was determined as 17.83 ± 0.17% (detailed results are provided as supporting information).

In this study, the effect of BPJ and the three fractions of *B. pinnatum* on the contractility of porcine detrusor strips in a tissue organ bath system were compared with oxybutynin.

At a test concentration of 10%, BPJ reduced the contractility of the detrusor to 58.6 ± 13.3% after 74 min, after a dose-independent initial increase in contractility during the first 40 min (Fig. 5). The flavonoid fraction, at 1 mg/mL, led to a maximum reduction of the contractility to 21.3 ± 5.2% after 77 min. The significant inhibitory effect was dose- and time-dependent, when compared to control strips ($p<0.0001$). As in the case of BPJ, the muscle strips showed an increased contractility in the first 35 min of the stimulation period (Fig. 6a). The bufadienolide fraction showed no inhibitory effect on the contractility at concentrations of 0.1 - 40 µg/mL. The muscle contractility became more intense with increasing bufadienolide concentration (Fig. 6b). The polar fraction reduced the contractility of the detrusor muscle strip in a reversible manner. A maximum reduction of the detrusor contractility to 15.5 ± 5.5% was achieved after 23 min ($p<0.0001$) (Fig. 7a). In experiments with L-malic acid (1.5 mg/mL) a similar time-effect curve as for the polar fraction (3 mg/mL) was obtained. It therefore seemed that L-malic acid significantly contributed to the inhibitory effect of the polar fraction (Fig. 7b). For a clarification of the mechanism involved, we performed pH measurements over a period of 74 min. The results suggested that the inhibitory effect of the polar fraction was due to a pH decrease from 7.4 to 4.5. The effect was reversed when the pH was increased to 7.4 (with NaOH) during the experiment. Also, we found that D-malic acid (1.5 mg/mL) and succinic acid (1.3 mg/mL, pH 5.4 in the chamber) both inhibited the contractility of muscle strips. Based on these experiments, we concluded that the inhibitory effect of the polar fraction was caused by a lowering of the pH.

The reference compound oxybutynin reduced at a 10^{-6} M concentration the detrusor muscle contractility to 21.9 ± 4.7% after 50 min ($p<0.0001$). A rapid inhibition of the detrusor contractility was observed as a consequence of the muscarinic receptor antagonism (Fig. 8).

Muscle vitality of the strips treated with the highest test concentrations was checked by further 9 stimulations after a washout period of 30 min. The muscle contractility at 20 min and 68 min, and at the end of the second stimulation period (recovery) is shown in figure 9. Muscle strips treated with BPJ recovered to 82.4% of contractility before treatment. The flavonoid fraction altered the muscle vitality irreversibly, as muscle strips only recovered to 32% of contractility. The muscle contractility after treatment with the bufadienolide fraction was still increased (121.3%) compared to the contractility before application. After treatment with the polar fraction, muscle strips recovered to 77.1% of initial contractility. In contrast, muscle strips treated with oxybutynin did not recover and showed a contractility of 19.5% at the end of the second stimulation period. The contractility of control strips treated with Krebs solution was only slightly reduced to 90.8% over the study period.

DISCUSSION AND CONCLUSION

Many individuals are affected by OAB, a heterogenous disease, and different, though not always satisfying treatment options are available. Treatment alternatives with phytopharmaceuticals such as *Bryophyllum pinnatum* are increasingly popular for patients with lower urinary tract disorders (Slavin et al., 2010). We here investigated the effect of BPJ, of flavonoid, bufadienolide, and polar fractions on the porcine detrusor contractility induced by EFS.

We observed an inhibitory effect of total BPJ on porcine detrusor contractility of 41.4%, and a significant dose- and time-dependent inhibition of the contractility by the flavonoid fraction of 78.7%. Hence, this fraction seems to play a major role in the inhibition of detrusor contractility by BPJ.

Normal bladder function includes the storage phase and the voiding/micturition phase. It is regulated through a complex efferent and afferent activity of parasympathetic, sympathetic, and somatic peripheral nervous systems (Abrams and Andersson, 2007). Recent studies have indicated locations where BPJ and flavonoids might alter bladder contractility. A previous study with BPJ (2%) showed an inhibition of the oxytocin-induced increase of $[Ca^{2+}]_i$ in human myometrial cells, which could be explained through a blockage of Ca^{2+} release from intracellular stores (Simões-Wüst et al., 2010). This study further showed that BPJ could delay voltage-dependent Ca^{2+}-influx through L-type Ca^{2+} channels. An inhibitory effect on porcine detrusor contractility through an inhibition of Ca^{2+} release from intracellular stores, and the involvement of L-type Ca^{2+} channels was described for the flavonoid galangin. Involvement of adrenergic and vanilloid receptors were ruled out (Dambros et al., 2005). Likewise, flavoxate (a synthetic flavone derivative) inhibits L-type Ca^{2+} channels in human detrusor (Tomoda et al., 2005).

Hence, modulation of intracellular calcium concentration seems to be a mechanism by which the flavonoid fraction exerts its activity on the detrusor muscle. An α_1-adrenoceptor antagonistic effect, as described for some flavonoids, could be an alternative mechanism which would need further investigations with *B. pinnatum* (Li et al., 2011).

All muscle strips treated with BPJ and flavonoid fraction showed an initial increase of contraction for about 35 min prior to relaxation phase. This can be explained by a bimodal effect, which has been reported for some flavonoids. For example, myricetin showed a potentiation of muscle contraction in vascular tissue when administered at low concentrations, and a stimulatory activity on L-type Ca^{2+} channels. With higher concentrations, however, myricetin had a relaxant effect on the smooth muscle (Fusi et al., 2003).

As expected, the bufadienolide fraction showed no inhibitory effect on porcine detrusor contractility. While bufadienolides inhibit the Na^+-K^+-ATPase in the kidney they are not known to affect the bladder wall directly (Puschett et al., 2010).

The polar fraction showed a reversible inhibitory effect on porcine detrusor contractility through a non-physiologic pH-drop in the organ bath, without any pharmacological influence on the muscle strips.

Our research indicates that the flavonoid fraction of *B. pinnatum* appears to be the pharmacologically relevant portion causing bladder muscle relaxation. While oxybutynin demonstrated a fast onset of relaxation to 21.9% after 50 min, the flavonoid fraction demonstrated an initial increase followed by a decrease in contractility to 21.3 % after 77 min. At a concentration of 1 mg/mL the flavonoid fraction showed a reduction in contractility comparable to oxybutynin at a concentration of 10^{-6} M. Further investigations are needed for a better understanding of the underlying molecular pathways.

Supporting information

HPLC-PDA-MS chromatogram and NMR analyses of the polar fraction of *B. pinnatum* are provided as supporting information.

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Conflict of Interest

M.M. and M.S. are employees of Weleda AG.

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Legends of Figures

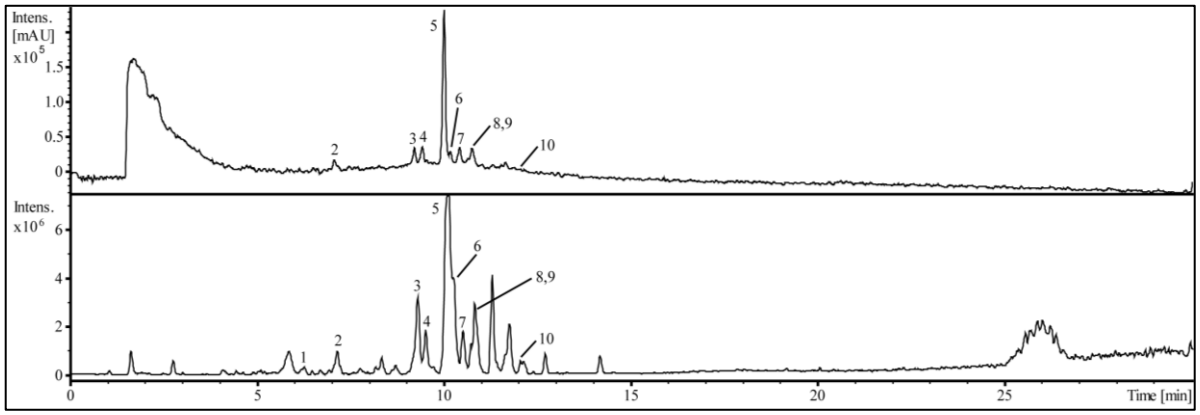

Fig. 1 HPLC-PDA-ESI-MS of the flavonoid fraction. Top: UV trace (200-700 nm). Bottom: ESIMS base peak chromatogram (positive ion mode, *m/z* 150-1500). Sunfire^{TM} C_{18} column, A: 0.1% aqueous formic acid and B: MeCN, 5-100% B in A in 30 min, 0.5 mL/min. Numbers refer to the isolated reference substances **1**-**10**.

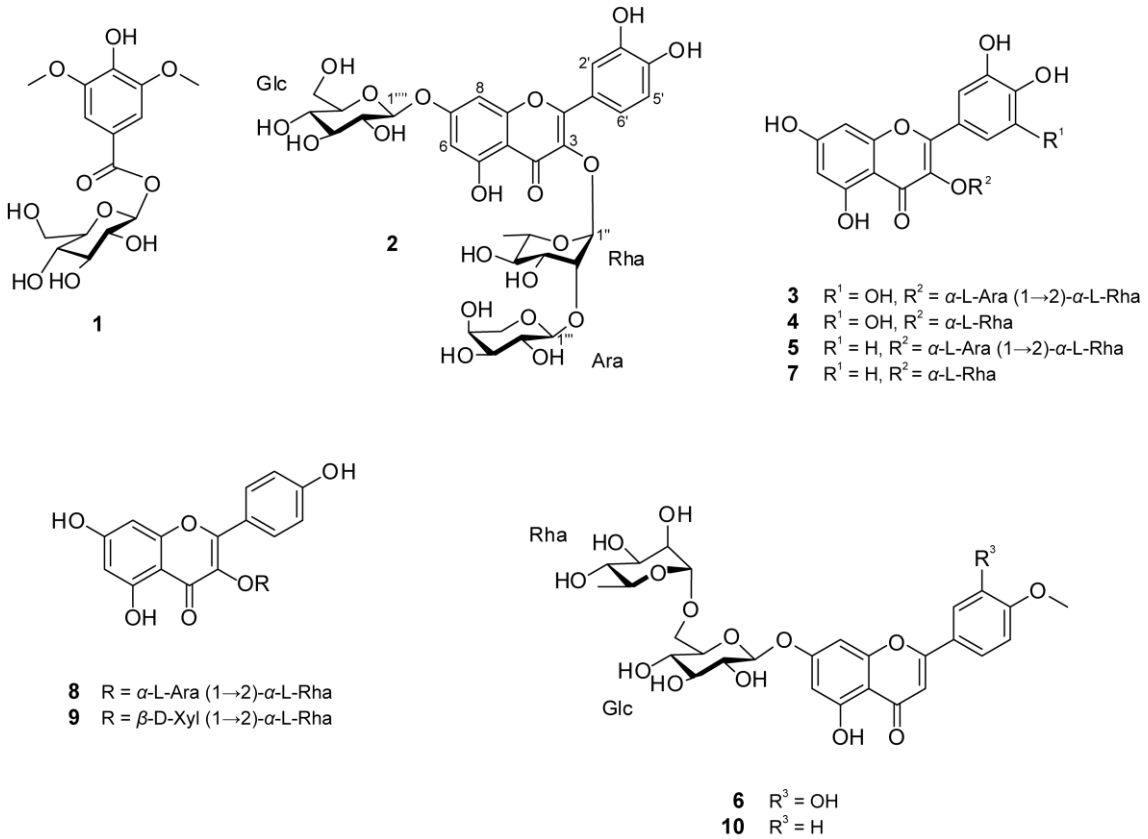

Fig. 2 A phenolic acid derivative (**1**) and nine flavonoid glycosides (**2-10**) contained in the flavonoid fraction.

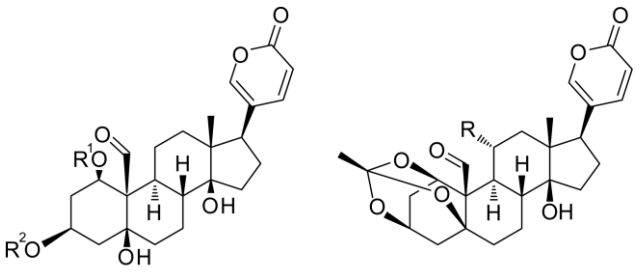

Fig. 3 Four bufadienolides (**11-14**) contained in the bufadienolide fraction.

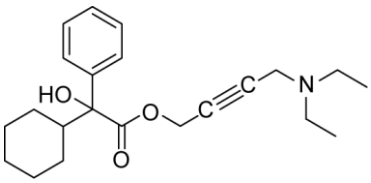

Fig. 4 Structure of oxybutynin.

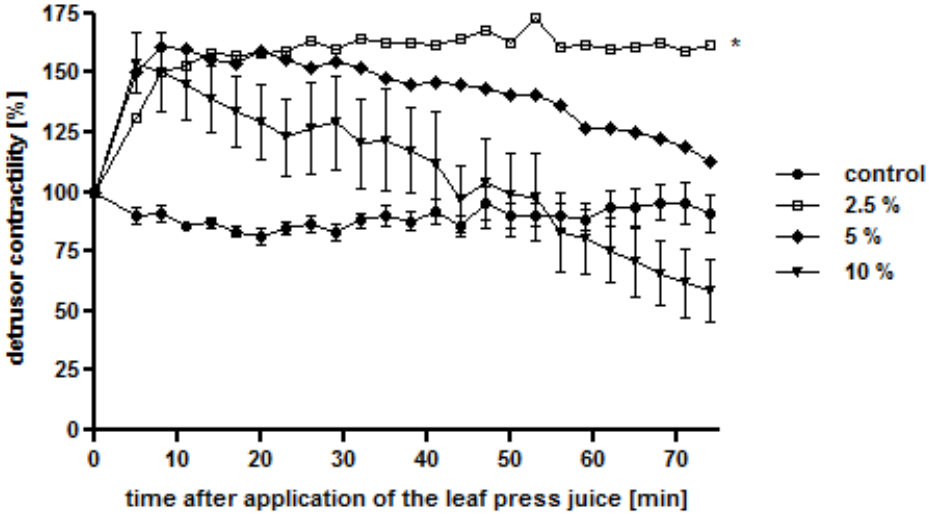

Fig. 5 Effect of *B. pinnatum* leaf press juice (2.5 - 10%) on porcine detrusor contractility. The maximum contractility before treatment was set as 100%. Krebs solution was used as control. Mean value ± SEM of n = 7 muscle strips are shown. *p < 0.05.

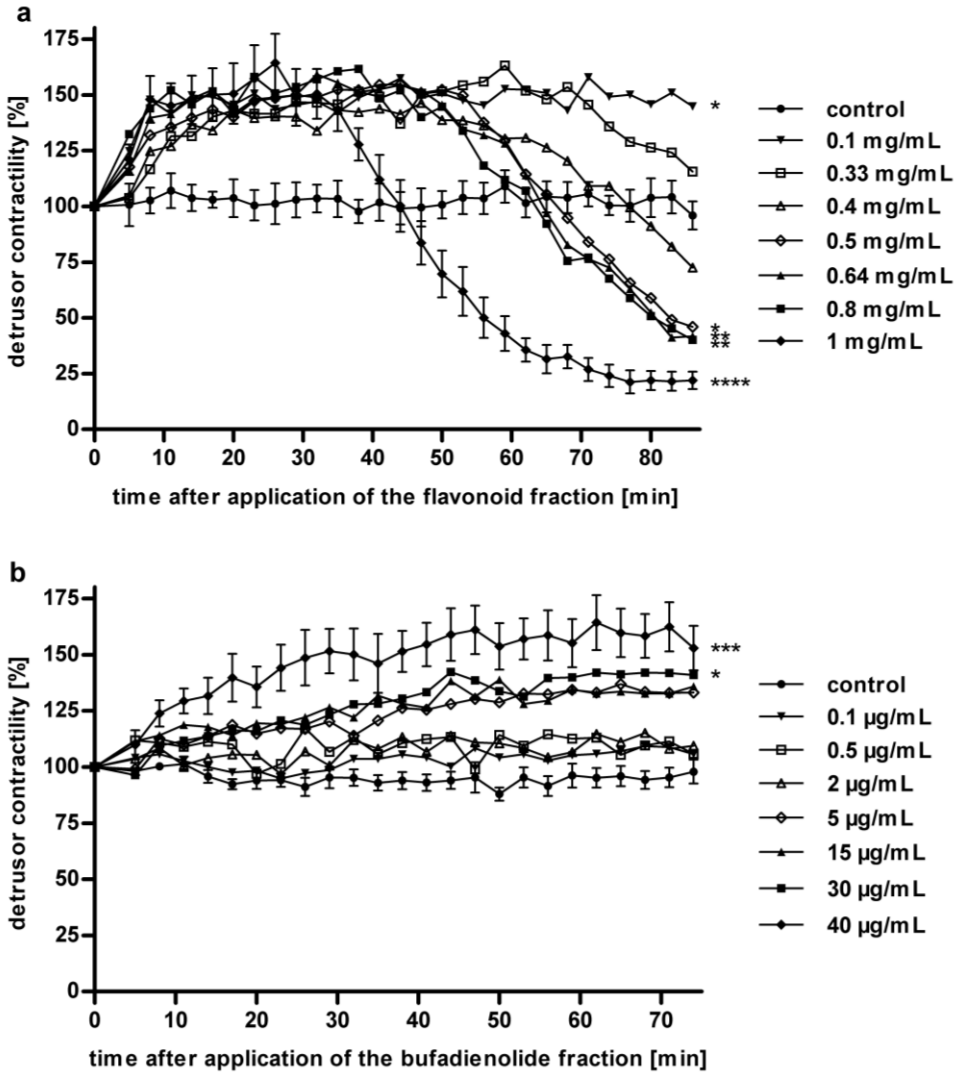

Fig. 6 (**a**) Effect of the flavonoid fraction (0.1 - 1 mg/mL) and (**b**) of the bufadienolide fraction (0.1 - 40 µg/mL), respectively, on porcine detrusor contractility. The maximum contractility before treatment was set as 100%. DMSO (0.5%) was used as control. Mean value ± SEM of n = 7 muscle strips are shown. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

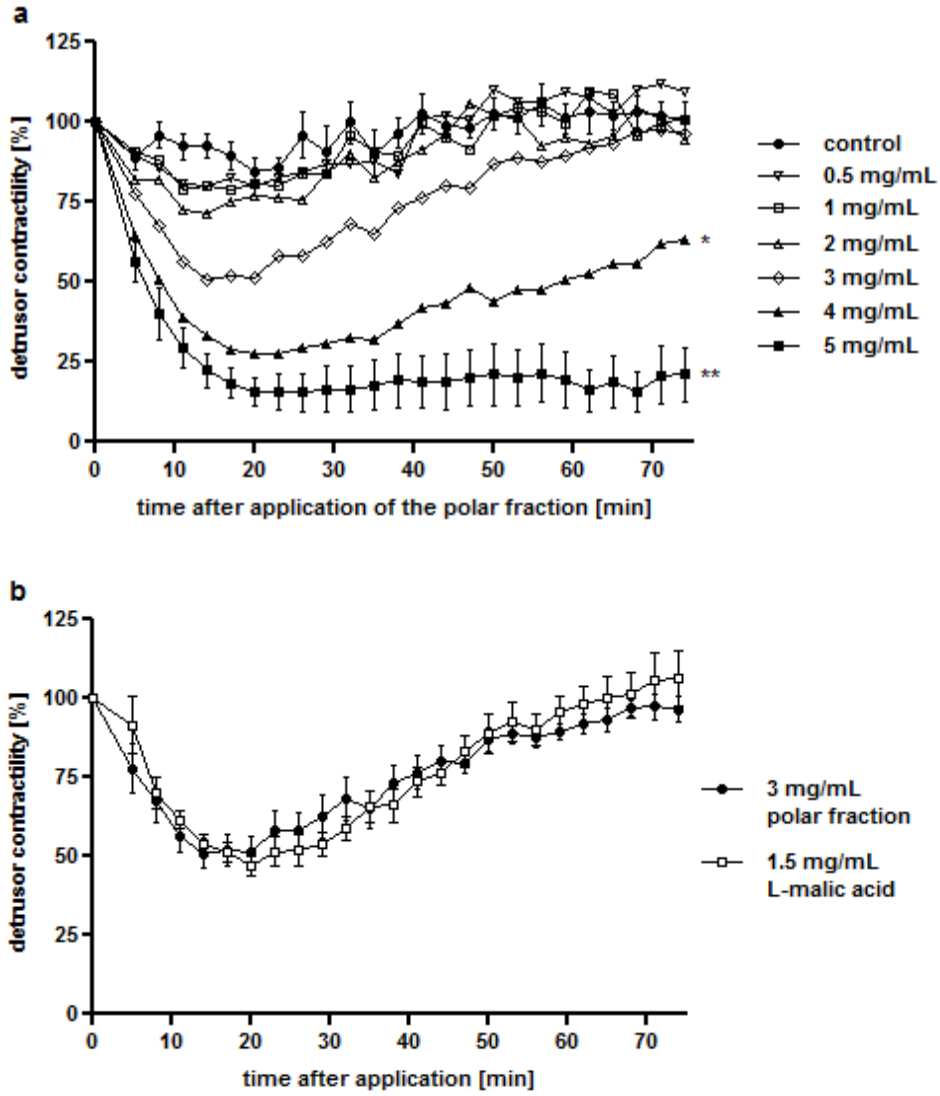

Fig. 7 Effect of the polar fraction and of L-malic acid, respectively, on porcine detrusor contractility. The maximum contractility before treatment was set as 100%. (**a**) The polar fraction (0.5 - 5 mg/mL) was tested, and Krebs solution served as control. (**b**) The effect of L-malic acid (1.5 mg/mL) was compared to the polar fraction (3 mg/mL). Mean value ± SEM of n = 7 muscle strips are shown. *$p < 0.05$ and **$p < 0.0001$.

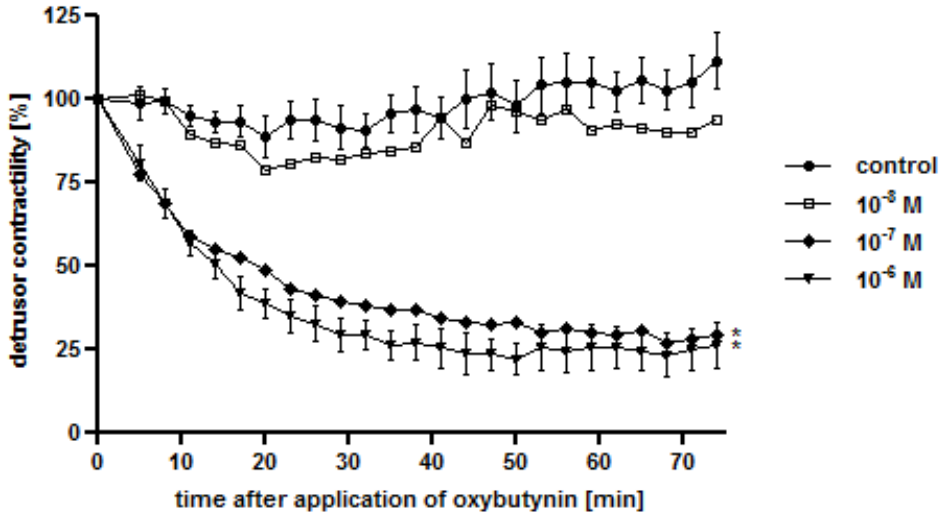

Fig. 8 Effect of oxybutynin (10^{-8} - 10^{-6} M) on porcine detrusor contractility. The maximum contractility before treatment was set as 100%. Krebs solution was used as control. Mean value ± SEM of n = 7 muscle strips are shown. *p < 0.0001.

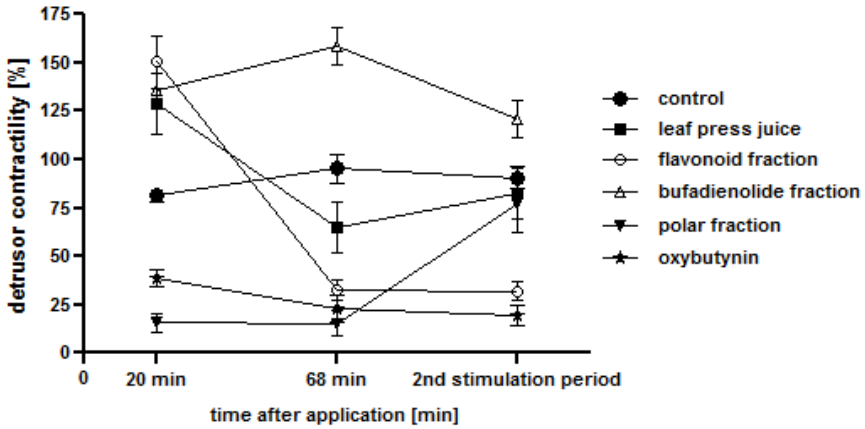

Fig. 9 Muscle contractility during the experiments. The contractility of the highest used concentration is shown after 20 min and 68 min, respectively, and at the end of the second stimulation period (recovery). Mean value ± SEM of n = 7 muscle strips are shown. Krebs solution was used as control.

Supporting Information

Inhibition of porcine detrusor contractility by the flavonoid fraction of *Bryophyllum pinnatum* – a potential phytotherapeutic drug for the treatment of the overactive bladder syndrome

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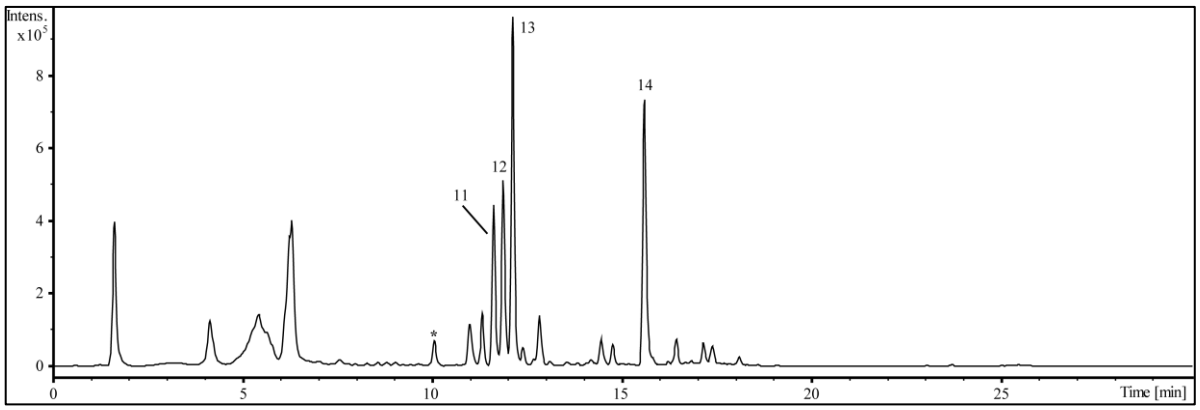
Legends of Figures

Fig. S1 HPLC-APCI-MS of the bufadienolide fraction. 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 the isolated reference substances **11-14**. *This peak (*m/z* 475.3) was tentatively identified as bryophyllin C.

Fig. S2 HPLC-ESI-MS of the polar fraction. ESIMS 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.

Fig. S3 ^{1}H NMR of L-malic acid (A) and of the polar fraction (B) (500 MHz, DMSO-d_6).

Fig. S4 ^{1}H NMR of L-malic acid (A) and of the polar fraction with 1,3,5-trimethoxybenzene as IS (B) (500 MHz, DMSO-d_6).



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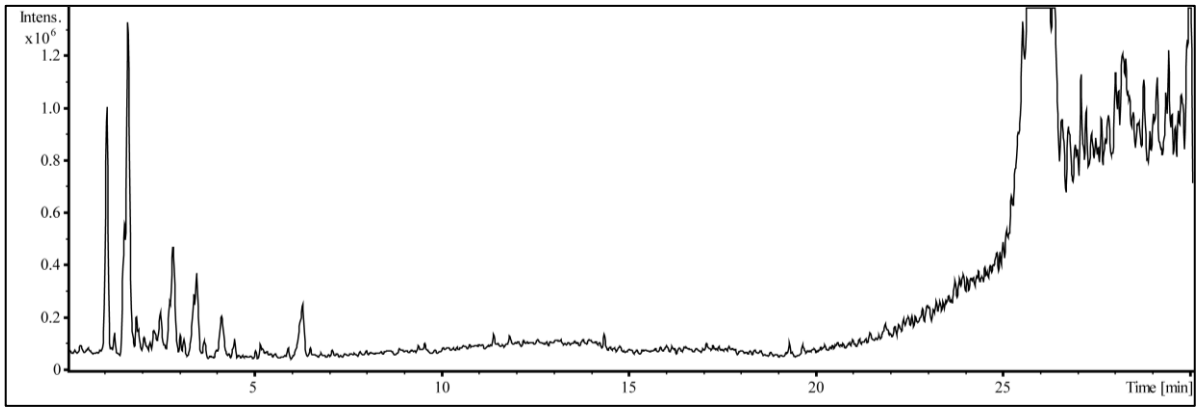

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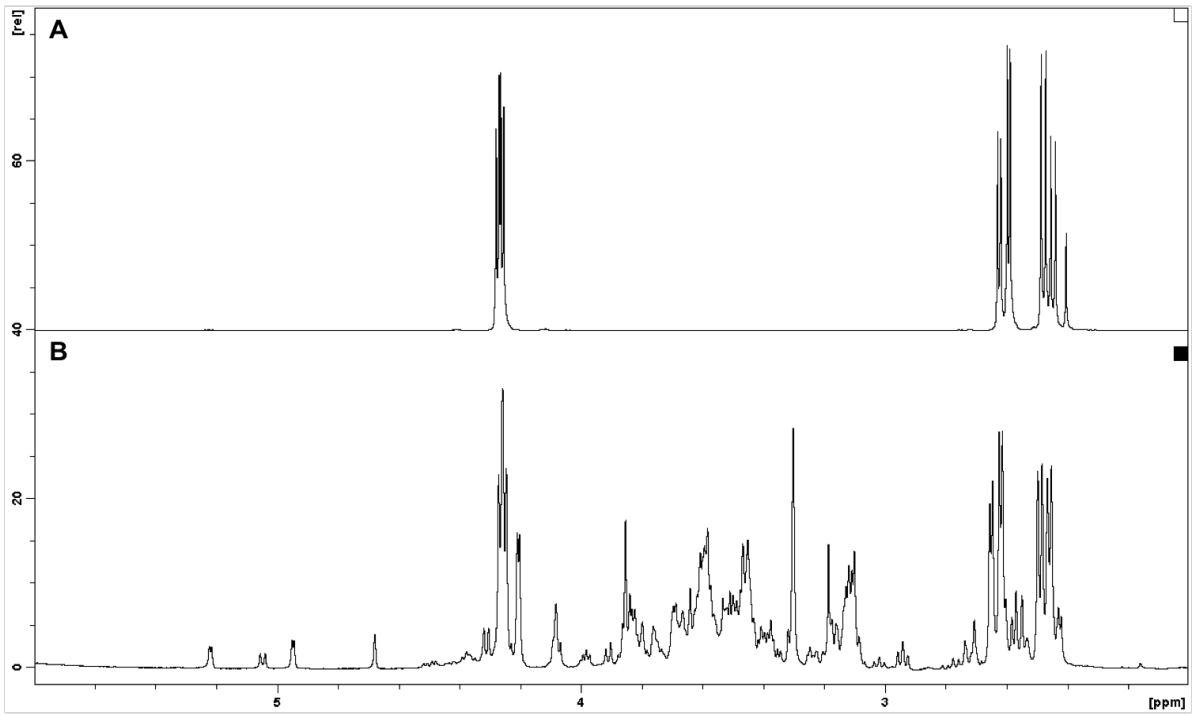

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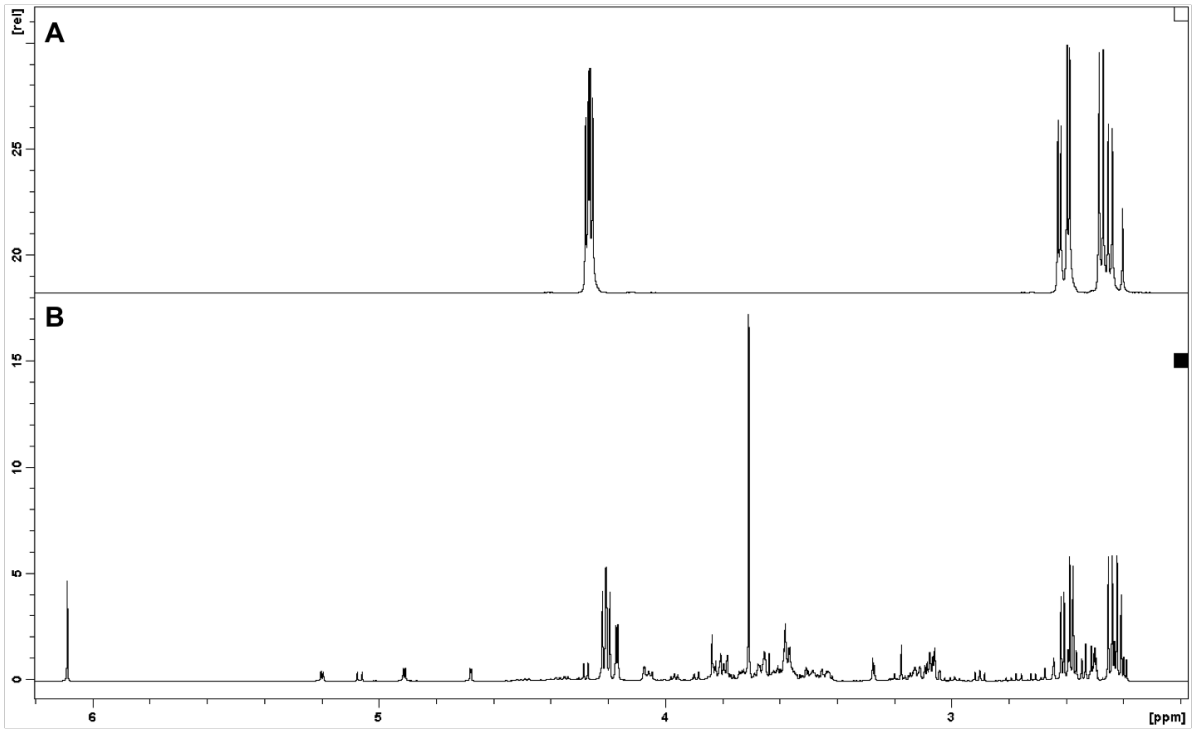

Fig. S4 ^{1}H NMR of L-malic acid (A) and of the polar fraction with 1,3,5-trimethoxybenzene as IS (B) (500 MHz, DMSO-d_6).

5 DISCUSSION AND OUTLOOK

5.1 Discussion

Bryophyllum pinnatum is a unique plant and a well-regarded phytotherapeutic with a potential to treat hyperactive disorders. Clinical trials documented a potent tocolytic effect of *B. pinnatum* that was further investigated using myometrial strips to elucidate possible mechanisms involved in the effect. Results of *in vitro* experiments demonstrated a dose-dependent inhibitory effect of an aqueous leaf extract on the spontaneous human myometrial contractions as well as an inhibition of oxytocin-stimulated contractions [1]. *In vitro* contractility measurements using myometrial strips assume that flavonoids may be involved in the muscle relaxant effect of *B. pinnatum*. The fraction containing bufadienolides did not change the contractility, whereas the constituents were only tentatively identified [2].

Based on the fact that *B. pinnatum* demonstrated efficient smooth muscle relaxant activities, investigation to examine a potential effect on urinary bladder smooth muscle was initiated. Patients suffering from overactive bladder would be appreciative of an alternative phytotherapeutic treatment. The positive effect of *B. pinnatum* on the micturition frequency/24 h compared with placebo was almost significant, although the number of participants was low [3]. These observations are promising and we therefore investigated the effect of *B. pinnatum* leaf press juice on porcine detrusor contractility *in vitro*. The leaf press juice demonstrated a relaxant effect on carbachol pre-contracted detrusor muscle strips and also an inhibitory activity on electrically induced muscle contractility [4]. Carbachol is a cholinergic agonist and stimulates muscarinic receptors. Therefore, the relaxation of carbachol-induced muscle contractility by press juice may indicate a competitive antagonistic effect of *B. pinnatum* on muscarinic receptors.

Phytoherapeutics often produce their therapeutic effects via the synergism of different constituents. An important aim of this thesis was to obtain basic knowledge concerning the phytochemical composition of *Bryophyllum pinnatum*. Two phenolic acid derivatives and nine flavonoid glycosides, including two new natural products, quercetin 3-O-*α*-L-arabinopyranosyl-(1 $\rightarrow$ 2)-*α*-L-rhamnopyranoside 7-O-*β*-D-glucopyranoside and myricetin 3-O-*α*-L-arabinopyranosyl-(1 $\rightarrow$ 2)-*α*-L-rhamnopyranoside, were isolated from the methanolic leaf extract of *B. pinnatum*. In addition, we detected L-malic acid as well as lysophosphatidylcholine derivatives in *B. pinnatum*. Concerning the safety of ingesting *B. pinnatum* preparations, we have investigated the presence of potentially cardiotoxic bufadienolides. We observed quantitative and qualitative differences between the two related species, *B. pinnatum* and *B. daigremontianum*. The latter is known to contain higher amounts of bufadienolides and also an additional prominent bufadienolide, daigremontianin. In the

methanolic extract of *B. pinnatum*, bufadienolides were not detectable using HPLC-PDA-ESIMS, due to their small amounts and low UV-activity. In a first instance, we needed to isolate reference substances from *B. daigremontianum* to subsequently detect bersaldegenin-1-acetate, bryophyllin A, bersaldegenin-3-acetate, and bersaldegenin-1,3,5-orthoacetate in *B. pinnatum* using HPLC-PDA-APCIMS. To our knowledge, up until now bersaldegenin-1-acetate had not been reported to be present in *B. pinnatum* [5]. These results led to the question, which amounts of bufadienolides have to be expected in *B. pinnatum* preparations? Recently, the quantification of these four bufadienolides was performed during a master thesis. *B. pinnatum* leaves from Brazil and Germany have been investigated and revealed 3.78 mg/100 g dry weight (DW) and 0.98 mg/100 g DW of bersaldegenin-1,3,5-orthoacetate, respectively. Moreover, a low concentration of 0.006 mg/100 g DW was detected in the dried powder (leaf press juice and lactose) from Germany, which is used by Weleda to produce *B. pinnatum* chewable tablets [6]. Based on the analytical results, we were able to confirm that flavonoids are the main UV-active constituents of *B. pinnatum*, whereas bufadienolides are assumed to be present only in trace amounts. The latter is supported by the low concentration found in the dried powder and the observation that *B. pinnatum* is well tolerated and produces only few and mild side effects in patients [7].

This thesis concentrated on the characterisation of the substance groups responsible for the muscle relaxant effect. The leaf press juice as well as the flavonoid, bufadienolide, and polar fractions, isolated from the methanolic extract, were tested on porcine detrusor muscle strips. Performing a different method than Schuler et al., the leaf press juice demonstrated an inhibition of electrically induced contractility of 41.4%. Additionally, we observed a dose- and time-dependent inhibitory effect for the flavonoid fraction reaching a maximum inhibiton of 78.7% comparable with oxybutynin, a standard synthetic anticholinergic drug [8]. This led to the suggestion that the flavonoids contained in *B. pinnatum* are important for the inhibitory effect on detrusor muscle contractility. Comparing the time-effect curves of the flavonoid fraction with that of oxybutynin, we conclude that the flavonoids may have no dominating effect on muscarinic receptors. However, until now the effect of *B. pinnatum* on muscarinic receptors has not been clarified. The bufadienolide fraction did not demonstrate an inhibitory effect at the tested concentrations; it may be assumed that there is no direct influence on the porcine bladder wall. The highly polar fraction unexpectedly inhibited contractility, which may be explained by a lower pH in the organ bath due to the presence of L-malic acid.
Initial *in vitro* contractility experiments with human bladder detrusor strips confirmed the inhibitory effect, we observed with porcine detrusor strips. Moreover, human detrusor muscle strips were more sensitive to *B. pinnatum* than porcine muscle strips.

If we summarize the *in vitro* and *in vivo* effects, *B. pinnatum* decreases the intensity of detrusor contractility and micturition frequency. These results characterise the potential of *B. pinnatum* to be a novel pharmacological option for OAB treatment.

Different molecular pathways may be involved in the mechanism of action of *B. pinnatum*. Several relevant investigations have previously been published concerning the receptors and signalling pathways that play a role in the inhibition of smooth muscle contractility by either *B. pinnatum* or other known flavonoids. By closely investigating the effect of the leaf press juice on human myometrial cells, an inhibition of the oxytocin-induced increase of $[Ca^{2+}]_i$ and a delayed Ca^{2+} influx via L-type Ca^{2+}-channels has been observed [9]. Concerning flavonoids, a similar effect was observed with the flavonol, galangin [10]. Supporting these findings, the synthetic flavone derivative, flavoxate, is assumed to have little inhibitory effect on L-type Ca^{2+}-channels [11]. Discussions have also indicated that flavonoids have an α_1-adrenoceptor antagonistic effect, which leads to muscle relaxation [12]. Also the inhibition of the activation of the MAPK pathway by a flavonoid has been discussed to be responsible for the relaxation of human bladder smooth muscle cells [13]. As another possible mechanism, reactive oxygen species (ROS) can stimulate ATP and acetylcholine release or mediate capsaicin-sensitive C-fibers, inducing bladder hyperactivity. Kaempferol has been reported to suppress oxidative stress in an *in vitro* cell model and reduce bladder hyperactivity in rats by downregulating ROS [14].

Theses previous investigations naturally lead to the question if there are the same targets for *B. pinnatum* in the myometrial and detrusor muscle. Currently used tocolytic drugs include β_2-adrenoceptor agonists, oxytocin receptor antagonists as well as off-label used calcium antagonists and prostaglandin synthesis inhibitors. In the urinary bladder, adrenoceptors are also expressed, whereas only β_3-agonists are approved as a treatment for OAB. α_1-Adrenoceptor antagonists are used to treat benign prostatic hyperplasia by relaxing prostatic and urethral smooth muscles [15]. Oxytocin receptors are not present in the bladder, they are only suggested to be involved in the tocolytic activity of *B. pinnatum*. For the treatment of OAB, flavoxate is administered and known to target L-type calcium channels. As mentioned before, *B. pinnatum* may affect calcium-dependent mechanisms. Furthermore, the role of prostaglandin (PG) and phosphodiesterase type 5 (PDE5) in the urinary bladder function is discussed. PG receptor antagonists as well as PDE5 inhibitors may be a novel therapy option for OAB in future [16].

5.2 Outlook

This PhD thesis focused on the analytical investigation of *Bryophyllum pinnatum* and its potential for a novel treatment for overactive bladder syndrome.

Currently, further *in vitro* contractility experiments with human detrusor muscle are ongoing to complete the dataset. Due to our eliminatory criteria, only a few bladder wall samples could be included until now.

A further aim is the examination of molecular mechanisms responsible for the observed inhibitory effect on detrusor muscle contractility, including investigations on the cellular level. It is still not clarified, how the identified constitutents of *B. pinnatum* reach their targets in the urinary bladder. Therefore, the investigation of pharmacokinetic parameters would be of interest.

The efficacy of *B. pinnatum* for the treatment of OAB is further investigated based on the previous pilot study performed by Betschart et al. [3]. A prospective, multicentre, randomised, double-blind, placebo-controlled crossover phase III study is currently being conducted to confirm the observed therapeutic effect of *B. pinnatum*. The efficacy of *B. pinnatum* treatment in OAB is being verified against the selective antimuscarinic drug, solifenacin, and placebo [17]. Until now, only women were included in clinical studies and it would be interesting to identify possible gender differences. Besides that the therapeutic effect might also depend on the patients' age due to known changes in bladder tissue and receptor expression [18].

Bryophyllum pinnatum showed a great potential for the treatment of overactive bladder syndrome. Besides hyperactive disorders, preparations are prescribed for a broad spectrum of other indications [19]. Here, it will also be essential to examine the efficacy in clinical studies.

5.3 References

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“Vorwärts und rückwärts ist die Pflanze immer nur Blatt”

Goethe

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