





## *Bryophyllum pinnatum* modulation of signaling pathways relevant for preterm labor in human myometrial cells

Leonie Zurfluh<sup>a,b</sup>, Stefanie Santos<sup>a</sup> , Annina Ruppen<sup>a,c</sup>, Johannes Mosbacher<sup>d</sup> ,  
Christian Haslinger<sup>a,b</sup>, Nicole Ochsenbein-Kölble<sup>a,b</sup>, Olivier Potterat<sup>c</sup> ,  
Ana Paula Simões-Wüst<sup>a,b,e,\*</sup> 

<sup>a</sup> Department of Obstetrics, University Hospital Zurich, Zurich, Switzerland

<sup>b</sup> University of Zurich, Zurich, Switzerland

<sup>c</sup> Division of Pharmaceutical Biology, University Basel, Basel, Switzerland

<sup>d</sup> Institute of Pharma Technology, School of Life Sciences, University of Applied Sciences and Arts Northwestern Switzerland FHNW, Muttenz, Switzerland

<sup>e</sup> Klinik Arlesheim, Arlesheim, Switzerland

### ARTICLE INFO

#### Keywords:

*Bryophyllum pinnatum*  
Myometrium  
Oxytocin  
Cell signaling  
Bufadienolides  
Proteomics

### ABSTRACT

Preparations of *Bryophyllum pinnatum* have been used as a well-tolerated treatment of preterm labor, initially in anthroposophic hospitals and, more recently, also in conventional settings. *In vitro* studies with human myometrial cells have shown that *B. pinnatum* leaf press juice inhibits both intracellular Ca<sup>2+</sup> signaling and the activation of inflammatory pathways induced by the relevant hormone oxytocin. However, the compounds responsible for these inhibitory effects and the potential involvement of related signaling pathways remain unknown. In the present study, we aim to address these knowledge gaps. *In vitro* experiments were conducted in hTERT-C3 human myometrial cells, using alamarBlue assay, fluorescent intracellular Ca<sup>2+</sup> assay, ELISA, proteomics and real-time PCR. Contractility studies were conducted in an *ex vivo* organ bath model using human myometrial tissue. No single compound from *B. pinnatum* leaves mimicked the inhibitory effect of the whole leaf press juice on OT-induced Ca<sup>2+</sup> signaling. However, a bufadienolide-enriched fraction and the bufadienolides bersaldegennin-1,3,5-acetate, bryophyllin A and bersaldegennin-3-acetate, but not bersaldegennin-1-acetate, reduced OT-induced COX-2 expression and attenuated NFκB activation. That the juice can inhibit prostaglandin F<sub>2α</sub>-induced contractions was shown in the myometrium bath model. Proteomics analysis revealed that the leaf juice reduced expression of various extracellular matrix proteins. Cell viability assays showed that the various inhibitory effects cannot be attributed to cytotoxicity. Taken together, these results further support investigations on the use of *B. pinnatum* as a well-tolerated candidate for long-term treatment of preterm labor.

### 1. Introduction

Preterm birth, defined as birth before completion of 37 weeks of gestation, affects more than one in ten newborns worldwide [1,2]. Preterm birth can result in death, compromised health, and impaired neurologic, cognitive and behavioral development in children, leading, besides the untold distress, to significant socioeconomic effects [3–5]. Premature activation of myometrial contractility, cervical ripening and fetal membrane activation are the most important triggers for premature birth [6–8]. Pharmacological therapy primarily addresses premature myometrial contractions by using tocolytic agents. While all tocolytics are probably effective in delaying preterm birth compared with placebo

or no treatment for 2–7 days, thus ensuring enough time to improve fetal lung maturation and *in utero* transfer to a perinatal care center with an NICU [9], they do not seem to be suitable for longer maintenance tocolysis as they have fetal and maternal side effects without any evidence of providing a better outcome [10–12]. To date, none of the available treatment options effectively accomplishes the ultimate goal of keeping the fetus in the womb until term.

Although the complex and multifaceted mechanism of preterm labor is not yet understood, the hormone oxytocin (OT) is one of the main factors. Inflammatory processes also play an important role in the activation of term as well as preterm labor. During both events, inflammatory cytokines and chemokines are elevated and an increase in the

\* Correspondence to: University Hospital of Zurich, Department of Obstetrics, Sternwartstr 14/PF 125, LAB H1, Zurich 8091, Switzerland.

E-mail address: [anapaula.simoewuest@usz.ch](mailto:anapaula.simoewuest@usz.ch) (A.P. Simões-Wüst).

<https://doi.org/10.1016/j.bioph.2025.117919>

Received 21 November 2024; Received in revised form 10 February 2025; Accepted 14 February 2025

Available online 20 February 2025

0753-3322/© 2025 The Author(s). Published by Elsevier Masson SAS. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

expression of COX-2 followed by an increased production of PGF<sub>2α</sub> takes place [13,14]. Besides triggering contractions directly by stimulating Ca<sup>2+</sup> release from intracellular stores, OT can also activate pro-inflammatory pathways. In gestational tissue, treatment with OT leads to activation of NFκB and MAPK, followed by upregulation of key inflammatory labor-associated genes like IL-8, CCL5, IL-6 and COX-2 *in vitro* [15]. This is also supported by the OT receptor antagonists atosiban and nolasiban inhibiting contraction and pro-inflammatory responses in myometrium induced by OT and PGF<sub>2α</sub> *in vitro* [16]. PGF<sub>2α</sub> itself leads to an activation of NFκB and MAPKs in primary myometrial cells, and the increase in PGF<sub>2α</sub>-induced COX-2 expression is mediated by the FP receptor coupling to both G<sub>oq</sub> and G<sub>oi</sub> proteins [17].

The succulent plant *Bryophyllum pinnatum* is used for a wide range of indications in ethnomedicine, including insect bites, treatment of wounds, inflammatory conditions and gastrointestinal disease [18–20]. Noteworthy constituents are rather ubiquitous flavonoids and bufadienolides, a class of compounds more characteristic of the genus *Bryophyllum* [21]. In Europe, preparations of the plant have been used in the treatment of preterm labor, especially as add-on treatment. Research on the mode of action of *B. pinnatum* showed that the juice reduces the intensity of spontaneous contractions in human myometrial biopsies, with a fraction enriched in bufadienolides exerting a particularly strong effect [22–24]. *In vitro* studies with human myometrial cells revealed that *B. pinnatum* leaf juice (BPJ) inhibits OT-induced Ca<sup>2+</sup> signaling [25] and flavonoids as well as bufadienolides seem to be involved synergistically [26].

In view of the anti-inflammatory effects reported in ethnomedicine, researchers have investigated possible corresponding pharmacological effects of *B. pinnatum*. In a recent systematic scoping review, we retrieved 33 *in vitro* and *in vivo* studies on the anti-inflammatory and anti-nociceptive effects of *B. pinnatum* [27]. Furthermore, BPJ showed an inhibitory effect on the OT-triggered pro-inflammatory signaling cascade (MAPK activation) in human myometrial cell lines, with bufadienolides appearing to be mainly responsible for the effect [26].

However, it is not yet fully clear which constituents are responsible for the observed effects of *B. pinnatum* on OT-induced signaling in myometrium models, nor whether other regulatory mechanisms are involved. In this study, we aimed at: (1) identifying single compounds relevant for the inhibitory effect on OT-induced intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) increase, (2) investigating the effect of juice, fractions and single compounds on the OT-induced inflammatory pathway, (3) exploring the effect of press juice on other related signaling pathways, and (4) studying its impact on gene expression.

## 2. Methods

### 2.1. Plant material

Leaves of *B. pinnatum* originated from cultures at Weleda Brazil; a voucher specimen (ZSS 29717) was deposited at The Zurich Succulent Plant Collection. *B. daigremontianum* was provided by Klinik Arlesheim AG (Switzerland); a voucher specimen was deposited at the Division of Pharmaceutical Biology, University of Basel (Nr. 838).

Experiments were performed either with fresh or lyophilized BPJ resuspended in DMSO as indicated in each case. BPJ has previously been characterized [25,28].

#### 2.1.1. *B. pinnatum* fresh juice

The leaf juice was produced from leaves harvested in S. Roque, Brazil, on March 25, 2014. The juice was obtained by mechanical pressing of leaves in a roller mill. The procedure was identical to the initial steps of the protocol used for the production of the active ingredient of Weleda *Bryophyllum* 50 % chewable tablets (Weleda AG, Arlesheim, Switzerland; registered at Swissmedic). The juice was filtered using a 150 mm diameter paper filter (Schleicher and Schuell, Dassel, Germany), and aliquots were kept at -80°C until use. Experimental

concentration was 1 or 2 % of total press juice diluted in the appropriate experimental vehicle (buffer or culture medium).

#### 2.1.2. *B. pinnatum* press juice lyophilizate in DMSO

Leaves for the production of lyophilized juice were harvested in São Paulo, Brazil in March 2021. Juice was produced as described above and lyophilized in a three-phase process (freezing at -50°C; primary drying at -35°C, 100 mTor, 168 h; secondary drying at 20°C, 55 mTor, 72 h) in vacuum-sealed vials in a FTS Systems Dura-Dry MP Microprocessor Controlled Corrosion Resistant Freeze Dryer. A total of 147.5 g of fresh juice afforded 5.51 g of lyophilizate (3.7 % drying residue). For the experiments, the lyophilized juice was reconstituted in DMSO (room temperature; stock solution 50 mg/mL) and filtered with a 0.45 μm syringe filter. Aliquots were kept at -20°C. The concentration used in experiments was 100 μg/mL (which corresponds approximately to 0.25 % of fresh press juice).

#### 2.1.3. Bufadienolide- and flavonoid-enriched fractions

Fractions were prepared as previously described [29,30]. In short, the MeOH extract from lyophilized leaves of *B. pinnatum* was partitioned between H<sub>2</sub>O and CH<sub>2</sub>Cl<sub>2</sub>. The aqueous phase was further fractionated on a Diaion HP20 (Supelco, PA, USA) cartridge. Elution with MeOH yielded the flavonoid-enriched fraction (FEF). After evaporation to dryness, the CH<sub>2</sub>Cl<sub>2</sub>-soluble fraction [30] was further purified on a RP-18 cartridge to yield the bufadienolide-enriched fraction (BEF) [29]. DMSO stock solutions of BEF and FEF were prepared (1.3 and 10.0 mg/mL, respectively) and kept at -20°C. Concentrations in experiments were 2.2 μg/mL (BEF) and 17.4 μg/mL (FEF).

#### 2.1.4. Single compound isolation

Flavonoids were isolated as previously described from *B. pinnatum* [28]; bufadienolides were isolated from *B. daigremontianum* [31] since this species contains higher amounts of the latter compounds. Their identity was confirmed by MS and NMR analysis before doing the experiments. Test concentrations were 1 μM for bufadienolides and 20 μM for flavonoids.

## 2.2. Drugs and reagents

OT and atosiban were purchased from Bachem (Switzerland), and both used at a final concentration of 100 nM, if not stated otherwise. DMSO was obtained from Sigma-Aldrich (USA).

## 2.3. Cell culture

Human myometrial telomerase reverse transcriptase cells (hTERT-C3) provided by M. Grãos (Biocant, Cantanhede, Portugal) were cultured at humidified 37°C and 5 % CO<sub>2</sub> in DMEM-F12 medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Cytiva, USA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Cells were trypsinized, passaged twice per week and used up to passage 35. All cell culture media were from Gibco, Thermo Fisher Scientific (USA) unless otherwise stated.

## 2.4. Cell viability

Myometrium hTERT-C3 cells were seeded in clear, flat-bottom 96-well plates at a density of 16'000 cells/well. At 24 h post-plating, medium was removed and fresh medium solutions containing test substances (BPJ 100 μg/mL, BEF 2.2 μg/mL, FEF 17.4 μg/mL, bufadienolides 1 μM and flavonoids 20 μM) were added and incubated for 6 h at 37°C. DMSO and Triton X-100 (Sigma-Aldrich, USA) served as negative and positive controls, respectively. After exposure to test substances, an alamarBlue™ cell viability assay was performed according to manufacturer's instructions (Thermo Fisher Scientific, USA). Briefly, cells were incubated with assay reagent (alamarBlue 10 %) for 4 h and

afterwards fluorescence (excitation: 530 nm, emission: 590 nm) was measured using FlexStationII, recorded by SoftMaxPro v5 (both Molecular Devices). Data were then exported to Microsoft Excel (v. 2016) for analysis. Cell viability is presented as percent of untreated condition.

### 2.5. Intracellular $Ca^{2+}$ concentration

Myometrium hTERT-C3 cells were seeded into 96-well clear, flat bottom microplates (Greiner, Austria) at 16'000 cells/well, one day before the experiment. In a first step, cells were loaded with 1  $\mu$ M Calbryte520 AM (AAT Bioquest, USA) diluted in HBSS assay buffer (HBSS with 10 mM HEPES, 1 mM  $MgCl_2$  and 2 mM  $CaCl_2$ , pH 7.4) at 37°C for 1 h, followed by a washing step before placing the plate in the reading chamber of the FlexStation3 (Molecular Devices, USA). Test substances were prepared in the same HBSS assay buffer in a 96-well V-bottom plate (Greiner, Austria). Fluorescence was measured at 525 nm for 60 s and test substances were added to the cell plate after baseline recording for 18–20 s. Approximately 5 min later, the agonist OT (MedChemExpress, USA) was added during a second read again for 60 s. For each test substance, at least three independent experiments containing four replicates were performed. Data were recorded with the software SoftMaxPro v7.1 (Molecular Devices, USA) at a sampling rate of 1.5 s and exported for analysis in Microsoft Excel (2016). Relative change of peak fluorescence over baseline ( $\Delta F/F$ ) values were calculated for each well.

### 2.6. Total COX-2 expression

Myometrium hTERT-C3 cells were seeded into 6-well plates (140'000 cell/well) two to three days before the experiment. After reaching about 80 % confluence, cells were treated with BPJ, fractions, or isolated bufadienolides and flavonoids combined with OT for 6 h, the time needed to obtain a clear-cut OT-induction of COX-2 expression (data not shown). As controls, 0.2 % DMSO alone and the OT receptor antagonist atosiban were used. After incubation time, cells were collected, lysed and cell lysate was stored at  $-80^\circ C$  until analysis. COX-2 quantification was done with an ELISA kit (PathScan Total COX2 Sandwich ELISA Kit; Cell Signaling Technology, USA) according to manufacturer's instructions.

### 2.7. NF $\kappa$ B-p65 phosphorylation

Two days before the experiments were performed, hTERT-C3 cells were seeded into 6-well plates (140'000 cell/well). The evening before the experiment, cells were starved overnight with medium containing only 1 % FBS. On the day of the experiment, cells were pretreated with the test substances or 0.2 % DMSO for 30 min. Cells were then stimulated with OT (100 nM) for 15 min, and stimulation was stopped by washing with ice-cold PBS followed by flash-freezing in liquid nitrogen. Afterward, plates were kept at  $-20^\circ C$  until further processing. For protein extraction, cells were lysed with a cell lysis buffer containing Halt™ protease and phosphatase inhibitor cocktail (both from Thermo Fisher scientific, USA). The protein concentration of samples was measured using Pierce™ BCA protein assay kit (Thermo Fisher scientific, USA) according to manufacturer's protocol. Proteins (diluted for equal loading) were separated in a Mini-Protean TGX Stain-Free Gels (4–20 % gradient) and transferred to a PVDF membrane using the Trans-Blot Turbo Transfer system (both Bio-Rad, USA) following manufacturer's instructions. Membranes were cut at 55 kDa and incubated in primary antibody (upper half in phospho-NF $\kappa$ B-p65, 1:1000; lower half in  $\beta$ -actin, 1:1000), overnight at 4°C, followed by incubation in horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000) at room temperature for 1 h the next day (all antibodies from Cell Signaling, Switzerland). Signal detection was done using Supersignal™ West Pico Plus Chemiluminescent Substrate (Thermo scientific, USA). Detection and quantification of band intensities was performed using FusionCapt Advance system (Vilber, Eberhardzell, Germany). Blotting of

membranes for  $\beta$ -actin enabled equal loading confirmation. For each substance tested, four independent cultures of hTERT-C3 cell line were used ( $n = 4$ ).

### 2.8. Myometrial contractility

Myometrial biopsies were prepared for contractility studies in the organ bath model as previously described [22,23]. In short, myometrial biopsies (5 g) were taken from women undergoing term elective caesarean section after informed written consent (ethics approval: KEK-ZH-Nr. 2014–0717, approval date 12.05.2015). The biopsy originated from the cranial edge of the uterotomy. In the lab, longitudinal muscle strips of approximately 15 x 2x1 mm were cut, placed under tensions and allowed to contract regularly. Then, test substances (atosiban 60 nM, BPJ 1 %) were added to the organ bath (pre-treatment) and thereafter the concentration of stimulating  $PGF_{2\alpha}$  (Cayman Chemical, USA) was continuously increased (10 nM, 100 nM and 1000 nM). Contractions of myometrial strips were recorded throughout the experiments by LabChart Pro 8.0.6 (ADInstruments, Oxford, UK) and analyzed using the peak analysis module. Area under the curve (AUC) and amplitude of each contraction were calculated. In each of the 10-min phases, average AUC and average amplitude were taken as measures for the strength of contractility, while the number of contractions was taken as frequency. Changes in contraction strength (AUC and amplitude) and frequency were then calculated as percentage of initial value (regular spontaneous contractions) (Fig. 4A).

### 2.9. Proteomics

#### 2.9.1. Sample Preparation

On day one, hTERT-C3 cells were seeded into transparent T75 culture flasks at a density of 750,000 cells/flask. After two days, cells were exposed to BPJ 2 % or PBS as control for 24 h. Cells were collected and the cell pellets were washed a total of three times (by aspirating the supernatant and resuspending the cell pellet in 10 mL PBS). The cells were then counted and approximately 1000,000 cells per condition were collected and stored at  $-80^\circ C$  for subsequent analysis of proteins. For each sample, proteins were extracted using a tissue homogenizer (TissueLyser II, Qiagen, Germany) and digested by using a commercial iST Kit (PreOmics) according to manufacturer's protocol.

#### 2.9.2. Liquid chromatography-mass spectrometry analysis

The peptides were washed, eluted, dried and re-solubilized in injection buffer (3 % acetonitrile, 0.1 % formic acid), peptide concentrations were estimated by means of a NanoDrop spectrophotometer, injection amounts normalized to an absorbance of 0.3 at 280 nm and subjected to LC-MS/MS analysis as previously described in [32].

Briefly, mass spectrometry analysis was done on an Orbitrap Fusion Lumos system with a Digital PicoView source, coupled to an M-Class UPLC. The solvent used in the analysis had two channels: channel A with 0.1 % formic acid and channel B with 0.1 % formic acid and 99.9 % acetonitrile. Samples were prepared by loading diluted peptides onto a C18 trap column and eluted through a gradient at a flow rate of 300 L/min. The mass spectrometer was set to data-dependent mode, capturing full-scan MS spectra within a 300–1500  $m/z$  range at a resolution of 120,000. MS/MS data was collected using quadrupole isolation and HCD fragmentation. The instrument operated with a rapid scan mode and excluded certain precursor masses from further selection. The acquired data were managed using a local LIMS system and deposited in the ProteomeXchange Consortium's PRIDE repository.

#### 2.9.3. Protein identification and label free protein quantification

The acquired raw MS data were processed by MaxQuant (version 1.6.2.3), followed by protein identification using the integrated Andromeda search engine (Cox and Mann, 2008) as described previously in [32]. In short, separate MaxQuant experiments were run for

each sample set, using the SwissProt human proteome database. Fixed and variable modifications included carbamidomethylation, methionine oxidation, and N-terminal acetylation. Trypsin was used as the enzyme, allowing for up to two missed cleavages. A false discovery rate of 0.01 for peptides and 0.05 for proteins was applied, with label-free quantification enabled. Results were validated in Scaffold software, requiring at least two peptides per protein. Only proteins with an adjusted  $p$ -value  $\leq 0.05$  and a  $\log_2$  fold change  $> \pm 1$  were considered significant.

Significantly up and downregulated proteins were subjected to STRING analysis to identify and characterize functional associations among the proteins and retrieve interacting genes/proteins (STRING Consortium, Version 11.0b, Switzerland).

### 2.10. Real-time PCR

Myometrial hTERT-C3 cells were seeded into 6-well plates (140'000 cells/well) on day one. After 24 h, test substances BPJ 2 % and PBS were added to fresh culture medium and cells were incubated for 24 h. Total RNA was isolated from hTERT-C3 myometrial cells using an extraction kit (RNeasy Mini Kit, Qiagen, Germany) according to manufacturer's protocol, and its concentration and purity were measured using NanoDrop One (Thermo Fisher Scientific, USA). cDNA was prepared from 1  $\mu$ g RNA using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Thermo Fisher Scientific, USA) according to manufacturer's protocol. Analysis of gene expression of COL1A1 (Hs00164004\_m1) and COL1A2 (Hs00164099\_m1), and the housekeeping enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs02758991\_g1) was done using TaqMan primers and TaqMan Fast Universal PCR Master Mix (all from Applied Biosystems, Thermo Fisher Scientific, USA). Quantitative real-time PCR was done with the Applied Biosystem 7500 Fast Real-Time PCR System and QuantStudio software (both from Thermo Fisher Scientific, USA). mRNA expression of COL1A1 and COL1A2 genes was normalized to GAPDH expression and relative quantification to untreated condition was calculated using the  $-\Delta\Delta C_T$  method.

### 2.11. Statistical analysis

All experiments were performed at least three times independently (see detailed indications in figure captions), and results are expressed as mean  $\pm$  standard error of mean (SEM). Statistical analysis was

performed using GraphPad Prism 10 Software. The Shapiro-Wilk test was used to confirm normal distribution and a paired  $t$ -test was applied to compare groups in all experiments, except in the contractility experiment (organ bath), where a 2way ANOVA Dunnett's multiple comparison test was conducted.

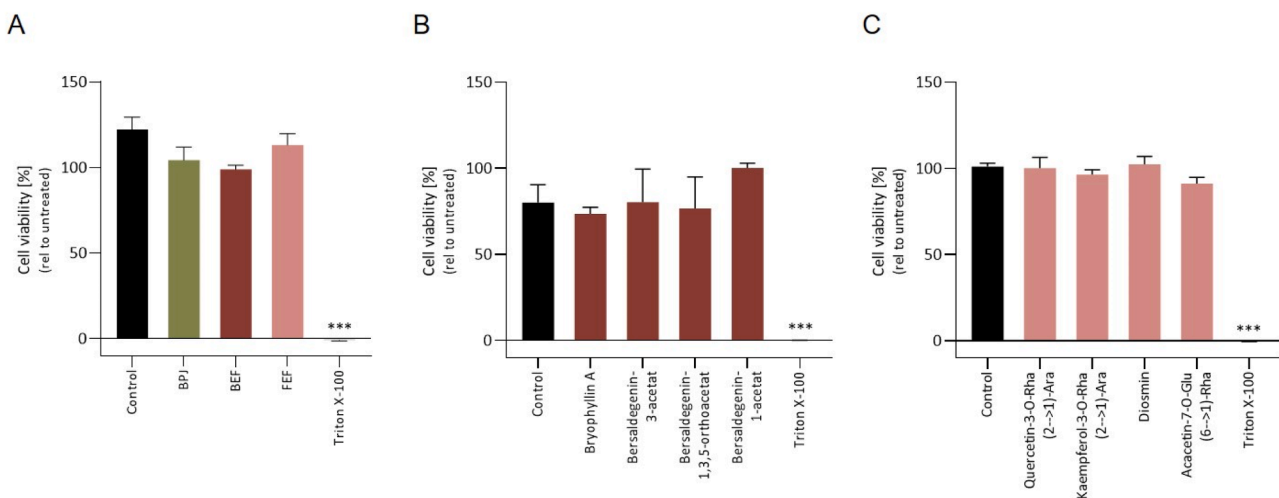
## 3. Results

### 3.1. BPJ, fractions and single compounds do not reduce cell viability of hTERT-C3 cells after 6 h

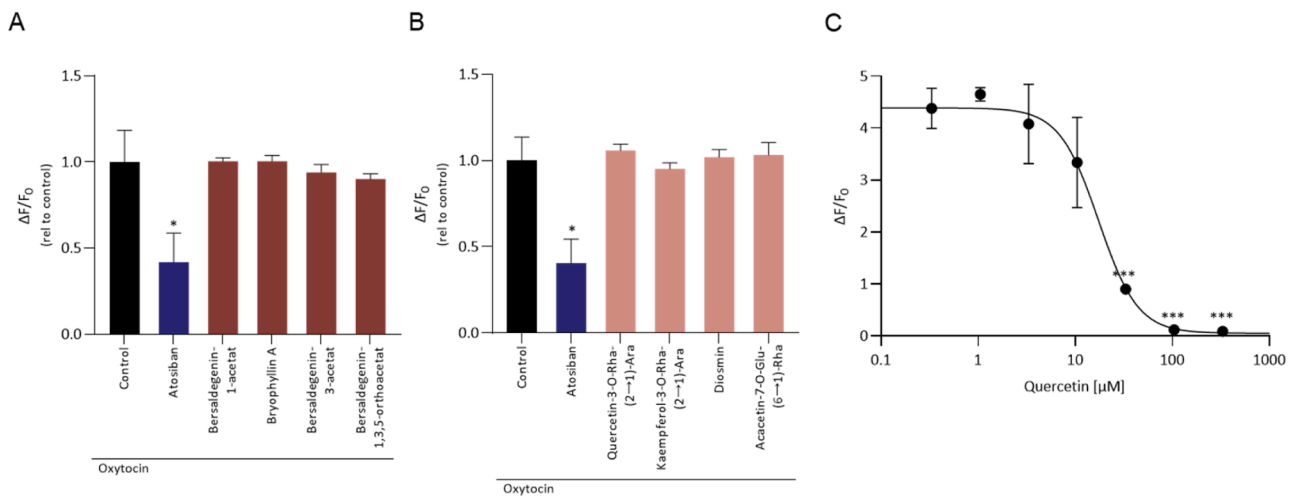
Fresh BPJ, fractions enriched in bufadienolides or flavonoids and the main flavonoid aglycones of *B. pinnatum* including quercetin (75 % of the aglycone fraction) have been previously shown not to diminish cell viability after 24 and 48 h up to concentrations higher than those typically used in experiments [22]. Before investigating the effects of lyophilized BPJ and isolated single compounds, we first assessed their cell tolerability in the alamarBlue assay. Given that the maximum exposure time in subsequent experiments was 6 h, this duration was tested. Cell viability did not significantly decrease in the presence of lyophilized press juice, fractions (Fig. 1A), bufadienolides (Fig. 1B), and flavonoids (Fig. 1C) compared with the DMSO control. Triton X-100 served as positive control and here cell viability was close to 0 %.

### 3.2. Isolated single compounds of *B. pinnatum* do not influence OT-induced intracellular $Ca^{2+}$ increase

We previously showed that BPJ inhibits the OT-induced increase of  $[Ca^{2+}]_i$  in myometrial cells [25,26]. In the present study, the goal was to identify the single compound(s) from BPJ that could be responsible for this effect. None of the four tested bufadienolides and flavonoids led to the inhibition of OT-induced increase of  $[Ca^{2+}]_i$  in our experimental set up (Fig. 2). For comparison, the effect of quercetin, the most common flavonoid aglycon in *B. pinnatum*, was investigated as well. In a concentration response experiment investigating the aglycon quercetin, marked inhibitory effects were apparent ( $IC_{50} = 25 \mu$ M; Fig. 2C). Several combinations of isolated bufadienolides and flavonoids did not show any inhibitory effect (Supplementary Figure 2).



**Fig. 1.** Effect of lyophilized *B. pinnatum* juice, fractions and single compounds on cell viability of hTERT-C3 cells after 6 h. Possible cytotoxic effects of (A) lyophilized press juice (BPJ 100  $\mu$ g/mL), the bufadienolide-enriched fraction (BEF, 2.2  $\mu$ g/mL), the flavonoid-enriched fraction (FEF, 17.4  $\mu$ g/mL), (B) isolated bufadienolides (1  $\mu$ M) and (C) isolated flavonoids (20  $\mu$ M) were investigated using the human myometrium cell line hTERT-C3. Viability assay was performed after 6 h: DMSO 0.2 % served as vehicle control; Triton X-100 served as positive control and data are expressed as percent viability of untreated cells. Data are presented as the mean  $\pm$  SEM  $\pm$  SEM of three independent experiments carried out in technical and biological triplicate ( $n = 3$ ). \*\*\* $p < 0.0005$  compared with control.



**Fig. 2.** Effect of *B. pinnatum* compounds on oxytocin-induced intracellular  $\text{Ca}^{2+}$  increase in human myometrial cells. Myometrial hTERT-C3 cells were treated with (A) bufadienolides (1  $\mu\text{M}$ ) and (B) flavonoids (20  $\mu\text{M}$ ) for 5 min before stimulation with OT (100 nM). Atosiban (100 nM) served as positive control, 0.2 % DMSO as vehicle control. Data are presented as the mean  $\pm$  SEM of three independent experiments carried out at least in technical quadruplicate ( $n = 3$ ); \* $p < 0.05$  compared to untreated condition. C: Concentration-response-curve of the flavonoid-aglycon quercetin inhibiting OT (100 nM) induced responses in hTERT-C3 cells.  $\text{IC}_{50}$  was determined at 25  $\mu\text{M}$ . Markers show average peak  $\Delta\text{F}/\text{F}$  fluorescent values; error bars SEM ( $n = 4$ ); \* $p < 0.05$ , \*\*\* $p > 0.0001$  compared with untreated control.

### 3.3. Bufadienolides modulate OT-induced COX-2 expression in human myometrial cells

Previous research showed that BPJ not only reduces the OT-induced increase in  $[\text{Ca}^{2+}]_i$ , but also attenuates the inflammatory pathway activated by OT. More specifically, BPJ and BEF were shown to inhibit the OT-induced activation of MAPKs, including SAPK/JNK and ERK1/2 [26]. Since COX-2 is one of the key pro-inflammatory enzymes partially regulated by the activation of MAPKs and NF $\kappa$ B, the effect of test substances on the expression of this enzyme was investigated. Atosiban ( $p = 0.011$ ) and BEF ( $p = 0.013$ ), but neither BPJ nor FEF, reduced the expression of COX-2 (Fig. 3A). Out of the four investigated bufadienolides, bersaldehynen-1,3,5-acetate ( $p = 0.009$ ), bryophyllin A ( $p = 0.033$ ) and bersaldehynen-3-acetate ( $p = 0.048$ ) led to a significant reduction of COX-2 expression at a concentration of 1  $\mu\text{M}$ , while bersaldehynen-1-acetate increased COX-2 expression ( $p = 0.049$ ) (Fig. 3B). One isolated flavonoid (quercetin-3-O- $\alpha$ -L-Ara-(1 $\rightarrow$ 2)- $\alpha$ -L-Rha) showed a slight tendency to increase the expression of COX-2 but did not reach significance ( $p = 0.088$ ; Fig. 3C).

### 3.4. Bufadienolides attenuate activation of NF $\kappa$ B-p65

Since the expression of COX-2 is also regulated by the activation of NF $\kappa$ B, we investigated the effects of BPJ, fractions, and single compounds on activation of NF $\kappa$ B subunit p65. In this experimental set up and using the same conditions as in the COX-2 cell experiment, a slight increase in phosphorylated NF $\kappa$ B-p65 upon treatment with OT was visible. After pre-treatment (30 min), we found that atosiban significantly attenuates phosphorylation of NF $\kappa$ B-p65 ( $p = 0.044$ ; Fig. 3D), as does BEF ( $p = 0.027$ ; Fig. 3D) as well as the isolated bufadienolides bryophyllin A and bersaldehynen-3-acetate ( $p = 0.019$  and  $p = 0.027$ , respectively; Fig. 3E). BPJ and bersaldehynen-1,3,5-orthoacetate showed a slight but non-significant inhibitory effect (Fig. 3D and E). Three of the tested flavonoids (diosmin, kaempferol-3-O- $\alpha$ -L-Ara (1 $\rightarrow$ 2)- $\alpha$ -L-Rha and acacetin-7-O- $\alpha$ -L-Rha (1 $\rightarrow$ 2)  $\beta$ -D-Glc) had a slight increasing effect that was not significant ( $p = 0.08$ ; Fig. 3E).

### 3.5. *B. pinnatum* press juice prevents PGF $_{2\alpha}$ -induced human myometrial contractions ex vivo

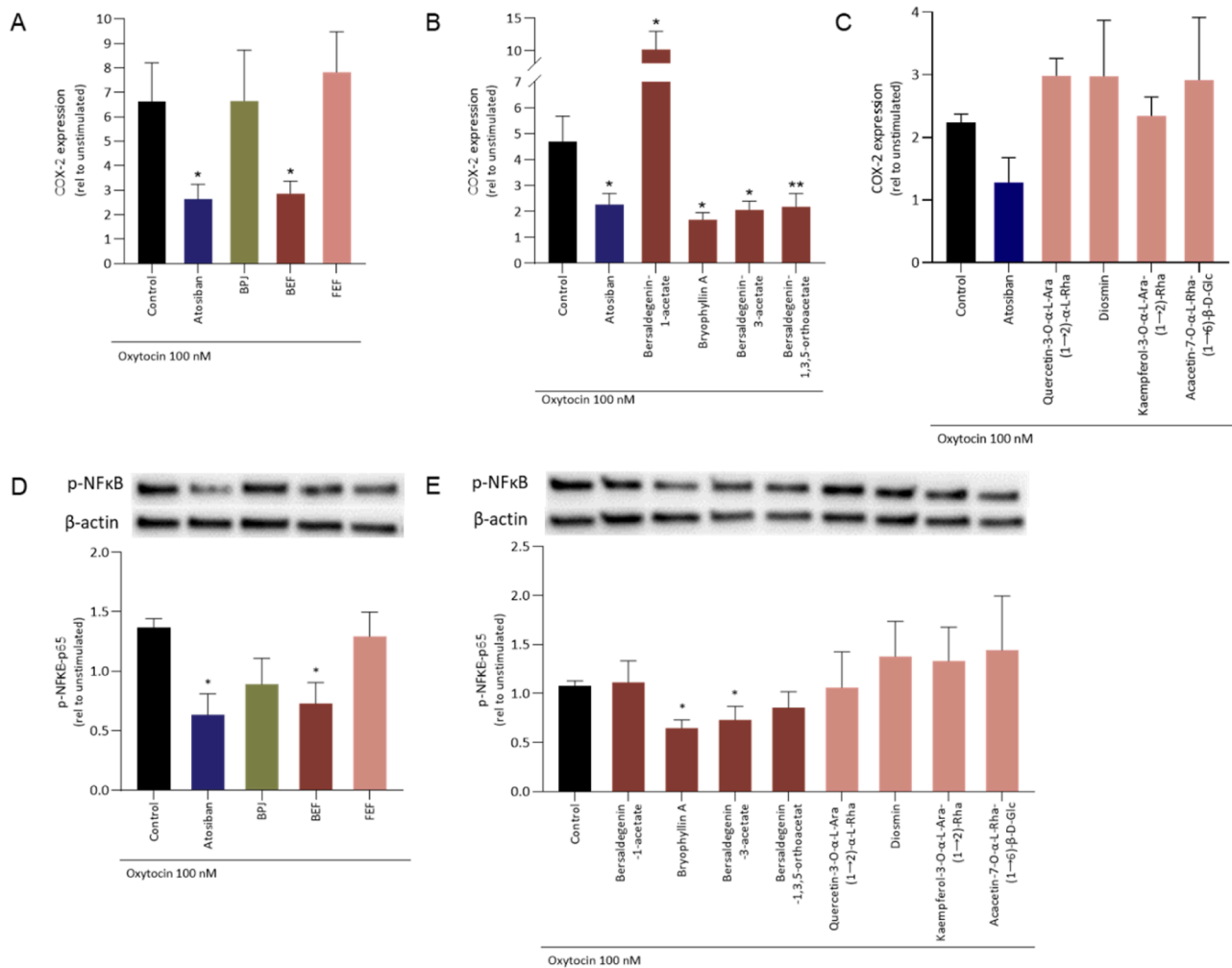
Probably due to the low expression of prostaglandin receptors in

hTERT-C3 cells, we were not able to induce an increase in  $[\text{Ca}^{2+}]_i$  with PGF $_{2\alpha}$  in our experimental set up mentioned above (real-time PCR and  $[\text{Ca}^{2+}]$  results; Supplementary Figure 1). Alternatively, the myometrium bath model was used. Here, a clear-cut PGF $_{2\alpha}$ -induced increase in contractility was observed (with increasing concentrations from 10 to 1000 nM). Pre-incubation of myometrial strips with atosiban 60 nM led to an inhibition of the PGF $_{2\alpha}$ -induced contractility, visible in the reduced increase of AUC compared with control (DMSO). At PGF $_{2\alpha}$  1000 nM, the AUC of atosiban-treated strips was at 70 % of control ( $p = 0.075$ ) (Fig. 4B). The amplitude of contractions in strips pre-treated with atosiban was also reduced, but only at the highest concentration of PGF $_{2\alpha}$  (Fig. 4C). Contraction frequency was not influenced by atosiban (Fig. 4D).

Pre-treatment of myometrial strips with BPJ 1 % fully prevented stimulation of AUC by PGF $_{2\alpha}$ , at 1000 nM with a statistical significance of  $p < 0.0001$  (Fig. 4B). Additionally, it significantly reduced contraction amplitude by 50 % ( $p = 0.0004$ ) (Fig. 4C). In line with previous observations [22,33], contraction frequency was almost five times higher in myometrial strips treated with BPJ 1 % ( $p < 0.0001$ ) (Fig. 4D).

### 3.6. *B. pinnatum* juice downregulates expression of extracellular matrix proteins (ECM)

To identify other potential regulatory effects of *B. pinnatum* in myometrium that might contribute to its reported tocolytic effect, we sought to characterize the influence of BPJ on the global protein profile of not otherwise stimulated human myometrial cells in culture. A proteomics approach led to the identification of 3093 proteins. After quantification, the variation in relative abundance between 2 % BPJ treated cells and control was determined. Table 1 displays a list of these proteins, with cutoff values set at  $\log_2\text{FC} > \pm 1$  and an adjusted  $p$ -value  $\leq 0.05$ . Eight proteins (0.3 % of total) were significantly upregulated, and 58 (1.9 %) were downregulated in the BPJ treated cells compared with PBS control (Fig. 5A). Proteins that differed in relative expression between the conditions were subjected to STRING functional network analysis, a tool that retrieves interacting proteins and predicts potential protein-protein interaction. For upregulated proteins, no functional association was found (Fig. 5B). In the list of the downregulated proteins, however, multiple proteins associated with the extracellular matrix (ECM) can be found, including COL1A2, COL1A1, COL5A1, COL4A1, FN1, and FBN1. Fig. 5C shows their functional relation. The detected



**Fig. 3.** Effect of *B. pinnatum* juice, fractions and compounds, and atosiban on oxytocin-induced COX-2 expression and NFκB activation. **A, B, C:** Myometrial hTERT-C3 cells were incubated with *B. pinnatum* test substances and OT (100 nM) for 6 h. DMSO 0.2 % and atosiban 100 nM serving as controls. Test substances included (A) lyophilized BPJ 100 μg/mL, corresponding concentrations of BEF (2.2 μg/mL) and FEF (17.4 μg/mL), (B) four bufadienolides (1 μM) and (C) four flavonoids (20 μM). Total COX-2 expression was measured by ELISA and represented relative to unstimulated condition (only DMSO 0.2 % without OT stimulation). **D, E:** In the phosphorylation experiments, hTERT-C3 cells were pretreated with (D) lyophilized *B. pinnatum* juice (BPJ; 100 μg/mL), bufadienolide-enriched fractions (BEF; 2.2 μg/mL), flavonoid-enriched fractions (FEF; 17.4 μg/mL); (E) bufadienolides (1 μM) and flavonoids (20 μM) for 30 min before stimulation with OT (100 nM) for 15 min. Control conditions were DMSO (0.2 %) and atosiban (100 nM). Whole cell proteins were subjected to western blot analysis with antibodies against phosphorylated NFκB-p65. Membranes were probed with β-actin to confirm uniform loading. Blot images are from a representative experiment and data are expressed normalized to β-actin and relative to unstimulated control (only DMSO 0.2 % without OT stimulation). Values represent the mean ± SEM of six (A), five (B, C, D) or four (E) independent experiments; \**p* < 0.05, \*\**p* < 0.01 compared to control.

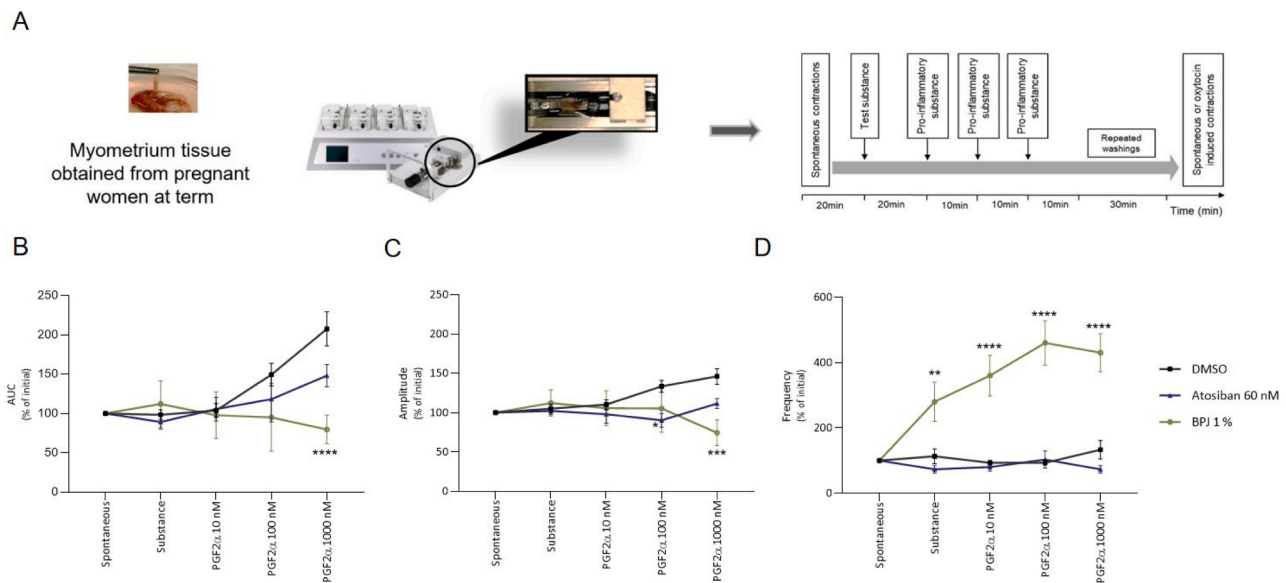
protein networks are associated with processes like skin morphogenesis, endodermal cell differentiation, cellular response and collagen metabolic processes (STRING analysis; Fig. 5D). To confirm the effect of BPJ observed in the proteomics approach, we investigated its effect on the expression of COL1A1 and COL1A2 (the two proteins that were most extensively downregulated) at mRNA level. BPJ reduced the expression of both genes by about 40 % (*p* = 0.011 for COL1A1 and *p* = 0.019 for COL1A2) (Fig. 5E).

## 4. Discussion

### 4.1. Effect of isolated single compounds of *B. pinnatum* on OT-induced intracellular signaling

In the present work, we tested four bufadienolides and four flavonoids isolated from *B. pinnatum* to identify the compound(s) responsible for the observed inhibitory effect of BPJ on OT-induced signaling

pathways. None of the investigated single compounds produced the observed inhibitory effect of BPJ on the OT-induced increase in  $[Ca^{2+}]_i$ . One possible explanation is that the combination of several compounds is necessary for the observed inhibitory effect of *B. pinnatum*. This assumption is based on previous findings showing that BEF and FEF exhibited a synergistic effect comparable to that of BPJ alone [26]. However, a combination of the isolated bufadienolides and flavonoids did not have the expected effect (data shown in Supplementary Figure 2), suggesting that one or more relevant compounds remain unidentified. We further attempted HPLC-microfractionation as an approach to detecting a potential unknown constituent responsible for the inhibitory effect. However, none of the microfractions led to significant inhibition (data not shown). Possible explanations are that compound concentrations may not have been sufficiently high in our assays, there could have been solubility problems, or a combination of compounds separated through the fractionation process may be necessary. Fractionation by preparative HPLC should be performed on a larger



**Fig. 4.** Effect of *B. pinnatum* juice and atosiban on prostaglandin  $F_{2\alpha}$ -induced myometrial contractions. A: In the experimental organ bath model for measurement of myometrial contractility *ex vivo*, myometrial strips were allowed to contract regularly, then increasing concentrations of prostaglandin  $F_{2\alpha}$  were added every 10 min in the presence or absence of test substances (BPJ 1 % or atosiban 60 nM) and contractility was recorded. A washout period of 30 min was then used to ensure tissue viability. B, C, D: The concentration of stimulating prostaglandin  $F_{2\alpha}$  was continuously increased (10 nM, 100 nM and 1000 nM). DMSO served as negative control. The graph shows (B) the area under the curve (AUC), (C) amplitude, and (D) frequency. Data was obtained with biopsies from five different patients ( $n = 5$ ) and is shown as percentage of stimulated probes compared with initial values. Results are presented as mean  $\pm$  SEM. Difference was analyzed with the Friedman test followed by Dunn's multiple comparisons test; \* $p \leq 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.0001$  compared to DMSO.

scale to allow testing at higher concentrations and the identification of unknown molecules of relevance as well as possible synergistic interactions in the future.

Herbal medicines are multi component complexes and it is often assumed that several components are necessary for an observed biological effect [34]. As the effects of the complete press juice are observed at relatively high concentrations (higher than 100  $\mu\text{g}/\text{mL}$ ), it appears likely that a single compound might not be sufficiently effective on its own. The relatively high Hill coefficient for the BPJ concentration-response curve on OT-induced  $[\text{Ca}^{2+}]_i$  signals in myometrial cells also indicates a cooperative action, either at multiple target sites or by multiple juice components [35]. Similar results were obtained when inducing  $[\text{Ca}^{2+}]_i$  signaling with the analogous peptide hormone vasopressin [35]. It is tempting to speculate that while some components could affect binding to the corresponding membrane receptors (OT and/or vasopressin), others could inhibit downstream events involved in triggering  $\text{Ca}^{2+}$  release from intracellular stores.

#### 4.2. Bufadienolides inhibit pro-inflammatory effects of OT in human myometrial cells

We confirmed previous findings [15,36] that OT activates inflammatory signaling pathways in human myometrium, as evidenced by the increase in COX-2 expression. The inhibitory effects of bryophyllin A, bersaldegenin-3-acetate and bersaldegenin-1,3,5-orthoacetate on OT-induced COX-2 expression align with previous data showing the inhibition of OT-induced activation of the inflammatory pathway by BEF through reduced phosphorylation of MAPKs JNK/SAPK and ERK1/2 [26]. These results are underlined by an inhibitory effect of bryophyllin A and bersaldegenin-3-acetate on NF $\kappa$ B-p65 activation, since the transcriptional factor also influences the expression of COX-2 and other inflammatory mediators [15].

The relevance of OT-driven activation of the COX-2-related pathway in the treatment of preterm labor is supported by the fact that COX inhibitors are considered "possibly effective" in the management of preterm labor [10]. In an *in vitro* study investigating the effect of the OT

antagonists atosiban and nolasiban on PGF $_{2\alpha}$ -stimulated contractility, the promising concept of using an OT antagonist for additional inhibition of inflammatory processes was proposed. Both tocolytics successfully inhibited PGF $_{2\alpha}$  induced contractility and suppressed the activation of pro-inflammatory pathways including NF $\kappa$ B, MAPKs and COX-2 in myometrium [16]. It is suspected that there is significant crosstalk between the OT receptors and other GPCRs relevant for myometrial contractility, such as the prostaglandin receptor [37]. As a next step, it would be interesting to investigate the effect of *B. pinnatum* compounds on the production of PGF $_{2a}$  *in vitro*, to demonstrate the physiologic relevance of the reduced expression of COX-2 mediated by bufadienolides.

The inhibition of COX-2 and the resulting reduction in prostaglandins is also an interesting therapeutic target for other pathologies related to uterine contractions, such as dysmenorrhea. In the dysmenorrheic uterus, high levels of prostaglandins, produced due to the increased availability of arachidonic acid from cell membranes in the shed uterine lining, are a significant contributing factor [38,39]. Given its anti-inflammatory and pain-reducing effects demonstrated *in vitro* and *in vivo*, *B. pinnatum* has recently been suggested as a potential treatment option for menstrual pain, based on available data in the literature and a case series involving five patients successfully treated with *B. pinnatum* (see [27] and references therein).

Compounds in medicinal plants can often be used as markers to standardize the production of plant extracts for herbal medication. Producing plant extracts enriched in certain compounds that either show profound therapeutic effects or unwanted effects is a common approach to optimizing the effectiveness and safety of herbal medications. In the case of *B. pinnatum*, candidates would be the biologically strongly active bufadienolides. Since one of the four investigated bufadienolides exhibited the opposite effect compared to the others, showing a significant increase in the OT-induced expression of COX-2, our work demonstrates that specific effects of the various individual bufadienolides should be considered.

**Table 1**List of significantly upregulated and downregulated proteins by *B. pinnatum* juice treatment of myometrial hTERT-C3 cells.

ID	Gene symbol	Gene name	Fold change	Adjusted p-value
<b>Upregulated</b>				
Q9ULX9	MAFF	transcription factor Maff	3.227	0.004
P02794	FTH1	ferritin heavy chain	2.962	0.015
Q13277	STX3	syntaxin-3	2.525	0.016
Q16763	UBE2S	ubiquitin-conjugating enzyme E2 S	2.301	0.008
P13747	HLA-E	HLA class I histocompatibility antigen, alpha chain E	2.049	0.009
Q9NZQ7	CD274	programmed cell death 1 ligand 1	1.694	0.046
Q96FZ7	CHMP6	charged multivesicular body protein 6 (chromatin-modifying protein 6)	1.469	0.047
Q13501	SQSTM1	sequestosome-1 (ubiquitin-binding protein p62)	1.203	0.017
<b>Downregulated</b>				
P08123	COL1A2	collagen type I alpha-2 chain	-7.750	0.00003
P02452	COL1A1	collagen type I alpha-1 chain	-6.745	0.00003
P15924	DSP	desmoplakin	-5.553	0.006
Q99715	COL12A1	collagen type XII alpha-1 chain	-5.545	0.005
P20908	COL5A1	collagen type V alpha-1 chain	-5.497	0.003
P02462	COL4A1	collagen type IV alpha-1 chain	-5.485	0.003
Q15014	MORF4L2	mortality factor 4-like protein 2 (transcription factor-like protein MRGX)	-4.393	0.019
O14530	TXNDC9	thioredoxin domain-containing protein 9 (ATP-binding protein associated with cell differentiation)	-4.203	0.006
P05997	COL5A2	collagen type V alpha-2 chain	-3.837	0.004
Q14118	DAG1	dystroglycan (dystrophin-associated glycoprotein 1)	-3.525	0.049
P82094	TMF1	TATA element modulatory factor (TMF)	-3.469	0.014
P09486	SPARC	secreted protein acidic and rich in cysteine (SPARC)	-3.451	0.00003
Q9NZJ9	NUDT4 DIPP2	diphosphoinositol polyphosphate phosphohydrolase 2 (DIPP-2)	-3.039	0.003
Q8NBJ4	GOLM1	golgi membrane protein 1	-3.028	0.038
P02751	FN1	fibronectin (FN)	-2.990	0.00011
Q15147	PLCB4	phospholipase C-beta-4 (PLC-beta-4)	-2.861	0.011
P04818	TYMS	thymidylate synthase	-2.811	0.037
P35442	THBS2	thrombospondin-2	-2.709	0.037
Q16270	IGFBP7 MAC25 PSF	insulin-like growth factor-binding protein 7 (prostacyclin-stimulating factor)	-2.642	0.00009
P35555	FBN1 FBN	fibrillin-1	-2.610	0.019
Q9UKI2	CDC42EP3	CDC42 effector protein 3	-2.442	0.046
O76021	RSL1D1	ribosomal L1 domain-containing protein 1	-2.412	0.016
P12109	COL6A1	collagen type VI alpha-1 chain	-2.394	0.009
P12110	COL6A2	collagen type VI alpha-2 chain	-2.363	0.00028
Q9UBF1	MAGEC2	melanoma-associated antigen C2	-2.293	0.009
O75976	CPD	carboxypeptidase D (Metalloprotease D)	-2.282	0.006
Q9P0P0	RNF181	E3 ubiquitin-protein ligase RNF181	-2.128	0.013
Q96EI5	TCEAL4	transcription elongation factor A protein-like 4 (TCEA-like protein 4)	-2.100	0.0407
Q6EMK4	VASN	vasorin	-2.055	0.015
Q9BZL1	UBL5	ubiquitin-like protein 5	-1.963	0.023
O94925	GLS	glutaminase kidney isoform, mitochondrial	-1.882	0.015
Q8NBT2	SPC24	kinetochore protein Spc24	-1.863	0.047
Q9BRK5	SDF4 CAB45	calcium-binding protein (Cab45) (stromal cell-derived factor 4) (SDF-4)	-1.832	0.001
P26358	DNMT1	DNA (cytosine-5)-methyltransferase 1	-1.777	0.008
Q53QV2	LBH	protein LBH	-1.737	0.019
P63218	GNG5	guanine nucleotide-binding protein subunit gamma-5	-1.720	0.019
O43805	SSNA1	sjoegren syndrome nuclear autoantigen 1	-1.704	0.041
Q9Y237	PIN4	peptidyl-prolyl cis-trans isomerase NIMA-interacting 4	-1.661	0.017
P46939	UTRN	utrophin	-1.592	0.016
P07711	CTSL	procathepsin L	-1.587	0.034
O15460	P4HA2	prolyl 4-hydroxylase subunit alpha-2	-1.556	0.003
O76031	CLPX	ATP-dependent Clp protease ATP-binding subunit clpX-like, mitochondrial	-1.553	0.004
P42892	ECE1	endothelin-converting enzyme 1	-1.539	0.006
Q12841	FSTL1	follicle-stimulating protein-related protein 1	-1.499	0.025
Q9Y697	NFS1	cysteine desulfurase, mitochondrial	-1.494	0.016
Q9BV57	AD11 MTCBP1	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	-1.447	0.019
P48960	ADGRE5 CD97	adhesion G protein-coupled receptor E5 (Leukocyte antigen CD97)	-1.444	0.006
Q8NFC6	BOD1L1	biorientation of chromosomes in cell division protein 1-like 1	-1.323	0.047
Q8TBA6	GOLGA5	golgin subfamily A member 5	-1.298	0.023
P23229	ITGA6	integrin subunit alpha-6 (CD49 antigen-like family member F)	-1.280	0.011
P08238	HSP90AB1	heat shock protein HSP 90-beta	-1.249	0.021
Q3KQU3	MAP7D1	MAP7 domain-containing protein 1	-1.236	0.037
O00754	MAN2B1	lysosomal alpha-mannosidase	-1.214	0.019
P11047	LAMC1	laminin subunit gamma-1	-1.208	0.008
P15586	GNS	N-acetylglucosamine-6-sulfatase (Glucosamine-6-sulfatase)	-1.131	0.009

(continued on next page)



been previously ignored. This approach revealed that BPJ causes a downregulation of ECM-related proteins. The downregulation of COL1A1 and COL1A2 was supported by the results of real-time PCR analysis. Collagens are essential, strength-providing ECM constituents that are not only relevant in the myometrium, but also in the structure of fetal membranes [43] and cervical smooth muscle cells [44]. The role of the ECM in various processes related to preterm birth is not yet fully understood and requires further investigation. However, it has been shown that collagen concentration increases with advancing gestation (a two-fold increase of collagen type I and III in the third trimester) [45]. Additionally, in animal models, myometrium and cervix collagen have been shown to undergo marked restructuring during pregnancy [46] and to increase in the laboring myometrium of rats [47]. Therefore, a reduction in expression of collagens and other ECM molecules in the – otherwise unstimulated – myometrium seems to support the use of *B. pinnatum* as a tocolytic. The modulation of ECM by *B. pinnatum* – at mRNA and protein level – is certainly slower and with delayed onset compared to its rapid effects on OT or NFκB signaling. These different dynamics need to be taken into account in future investigations. Our results – obtained with an immortalized myometrial cell line – should be first confirmed in different cells or tissue samples. Additional clinical conditions in which collagen reduction could be beneficial include delayed cervical ripening, assisted reproduction, to achieve appropriate endometrial microenvironment, and some phases of wound healing (see [48] and [49], respectively). Excessive ECM reduction in fetal membranes could have negative impact in pregnancy course. However, no evidence for such an effect was observed so far, despite the widespread use of *B. pinnatum* during pregnancy [40,50].

#### 4.5. General translational aspects

By revealing modulation of various signaling pathways relevant for preterm labor in human myometrial cells, our results seem to support previous studies on the therapeutic use of *B. pinnatum* preparations in tocolysis. In brief, a retrospective matched pairs study against standard treatment [51] led to two prospective, randomized trials – one for the prevention, and one for the treatment of premature contractions – which were terminated prematurely due to slow recruitment of participants [41]. Nevertheless, the results of the discontinued study on the acute treatment of premature contractions with *B. pinnatum* were promising enough to include *B. pinnatum* as the only herbal preparation among candidate substances with medium potential for preterm birth treatment in clinical development identified by the Accelerating Innovations for Mothers (AIM) database [52]. The good tolerability and safety of *B. pinnatum*, which has been reported in previous clinical studies [41, 53–57] and surveys [42,50], would support this candidate for preterm birth treatment. However, additional translational research is needed to confirm our observations in clinical settings.

Our previous work on myometrium contractility at the tissue level [23], point towards bufadienolides as class of molecules relevant for contractility inhibition. The same applies to previous and current data on inhibition of pro-inflammatory effects of OT by BPJ at the myometrium cell level (inhibition of MAPKs activation [26] and reduction of COX-2 expression). Bufadienolides exhibit a wide range of biological properties. They are present in several traditional remedies and their pharmacological activities have been extensively investigated [58].

A main limitation to the translation of our *in vitro* findings to clinical use is the lack of any pharmacokinetic studies for BPJ constituents to date. After oral application, they could undergo first-pass metabolism which may change their pharmacology. Nevertheless, it is worth mentioning that investigation of pharmacokinetic properties of different bufadienolides in the rat model after i.v. application revealed apparent elimination half-life values ranging from 18 to 80 minutes [59]. To put the existing data into context, although interspecies differences are likely, atosiban – which also inhibits the OT-induced increase of  $[Ca^{2+}]_i$  – has an estimated half-life of 18 minutes in humans [60]. Future studies

are needed to investigate the pharmacokinetics of bufadienolides from *B. pinnatum in vivo* and ideally in clinical trials. A short elimination time of bufadienolides speaks against relevant intestinal transformation; however, we can add that no changes in the similar molecule bufalin have been detected in the presence of intestinal microbiota of human origin (own unpublished observations). As for the highly abundant flavonoids, they are likely to be deglycosylated and transformed in the presence of intestinal microbiota [61]. A final limitation of our work is that the results were obtained using a transformed myometrial cell line, which might have different expression patterns and functional properties than *in situ* myometrial tissue exposed to hormones and embedded in a complex tissue matrix. While the used hTERT-C3 cell line offers the advantage of robust growth in culture, it does not reflect the exact situation pertaining in the myometrium of a pregnant woman.

## 5. Conclusion

In this study, we confirm and expand knowledge on the effect of BPJ on myometrial signaling pathways. Our findings provide deeper insights into the mechanism of action of herbal medications based on *B. pinnatum* by revealing the effect of single compounds (mainly bufadienolides) that reduce OT-induced COX-2 expression and attenuate NFκB activation in myometrial cells. Additionally, they demonstrate an impact on prostaglandin signaling pathways, which modulate myometrial contractions. Finally, we show that *B. pinnatum* also leads to the downregulation of ECM proteins.

Taken together, our findings further support research on *B. pinnatum* as a candidate tocolytic, and provide additional rationales for investigations regarding its application in other conditions with related pathologies, such as dysmenorrhea.

## Abbreviations

$[Ca^{2+}]_i$ , Intracellular calcium concentration; BEF, Bufadienolide-enriched fraction; BPJ, *Bryophyllum pinnatum* press juice; ECM, Extracellular matrix; FEF, Flavonoid-enriched fraction; HBSS, Hank's buffered salt solution; OT, Oxytocin; PG, Prostaglandin

## Funding sources

The author(s) declare financial support was received for the research of this article. This publication was supported partially by Weleda AG, the Johannes Kreyenbühl Foundation, the Dr. Hauschka Foundation, and the Association for Support of Anthroposophic Institutions. These funders, including the company Weleda AG, were not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

## CRediT authorship contribution statement

**Simões-Wüst Ana Paula:** Writing – review & editing, Supervision, Resources, Project administration, Methodology. **Zurfluh Leonie:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Ruppen Annina:** Writing – review & editing, Investigation. **Santos Stefanie:** Writing – review & editing, Methodology, Investigation. **Haslinger Christian:** Writing – review & editing, Methodology. **Mosbacher Johannes:** Writing – review & editing, Methodology. **Potterat Olivier:** Writing – review & editing, Methodology. **Ochsenbein-Kölbl Nicole:** Writing – review & editing, Resources.

## Declaration of Competing Interest

APSW has received research funding from Weleda AG over the last 5 years. The remaining authors declare that they have no conflict of interest.

## Acknowledgements

We thank the staff of the Department of Obstetrics at the University Hospital Zurich, who supported our study with the collection of myometrium samples. The authors would like to thank Bernd Roschitzki and Jonas Grossmann (Functional Genomics Center Zurich) for the protein analysis and data curation and Nadine Zeis-Kiprijanovski (Division of Pharmaceutical Biology, University of Basel) for the isolation of the bufadienolides. We further thank Denise Dawid for her support with experimental work. Katharina Gegenschatz Schmid and Deborah Spiess are gratefully acknowledged for their expertise in protein analysis. We further thank all members of the Bryophyllum Study Group for important scientific discussions and inputs.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2025.117919](https://doi.org/10.1016/j.biopha.2025.117919).

## References

- [1] E.O. Ohuma, A.B. Moller, E. Bradley, S. Chakwera, L. Hussain-Alkhateeb, A. Lewin, et al., National, regional, and global estimates of preterm birth in 2020, with trends from 2010: a systematic analysis, *Lancet* 402 (10409) (2023) 1261–1271, [https://doi.org/10.1016/s0140-6736\(23\)00878-4](https://doi.org/10.1016/s0140-6736(23)00878-4).
- [2] J.A. Martin, B.E. Hamilton, M.J. Osterman, *Births in the United States, 2021*, NCHS Data Brief. (442) (2022) 1–8.
- [3] J. Perin, A. Mulick, D. Yeung, F. Villavicencio, G. Lopez, K.L. Strong, et al., Global, regional, and national causes of under-5 mortality in 2000–19: an updated systematic analysis with implications for the Sustainable Development Goals, *Lancet Child Adolesc. Health* 6 (2) (2022) 106–115, [https://doi.org/10.1016/s2352-4642\(21\)00311-4](https://doi.org/10.1016/s2352-4642(21)00311-4).
- [4] M.A. Ream, L. Lehwald, Neurologic consequences of preterm birth, *Curr. Neurol. Neurosci. Rep.* 18 (8) (2018) 48, <https://doi.org/10.1007/s11910-018-0862-2>.
- [5] J.P. Vogel, S. Chawanpaiboon, A.B. Moller, K. Watananirun, M. Bonet, P. Lumbiganon, The global epidemiology of preterm birth, *Best. Pr. Res. Clin. Obstet. Gynaecol.* 52 (2018) 3–12, <https://doi.org/10.1016/j.bpobgyn.2018.04.003>.
- [6] N.E. Renthal, K.C. Williams, A.P. Montalbano, C.C. Chen, L. Gao, C.R. Mendelson, Molecular regulation of parturition: a myometrial perspective, *Cold Spring Harb. Perspect. Med* 5 (11) (2015), <https://doi.org/10.1101/cshperspect.a023069>.
- [7] M. Ilicic, T. Zakar, J.W. Paul, The regulation of uterine function during parturition: an update and recent advances, *Reprod. Sci.* 27 (1) (2020) 3–28, <https://doi.org/10.1007/s43032-019-00001-y>.
- [8] C.C. Ulrich, V. Arinze, C.B. Wandscheer, C. Copley Salem, C. Nabati, N. Etezadi-Amoli, H.R. Burkin, Matrix metalloproteinases 2 and 9 are elevated in human preterm laboring uterine myometrium and exacerbate uterine contractility, *Biol. Reprod.* 100 (6) (2019) 1597–1604, <https://doi.org/10.1093/biolre/iox054>.
- [9] R. Berger, H. Abele, F. Bahlmann, K. Doubek, U. Felderhoff-Müser, H. Fluhr, et al., Prevention and therapy of preterm birth. Guideline of the DGGG, OEGGG and SGGG (S2k-Level, AWMF Registry Number 015/025, September 2022) - Part 1 with recommendations on the epidemiology, etiology, prediction, primary and secondary prevention of preterm birth, *Geburtshilfe Frau* 83 (5) (2023) 547–568, <https://doi.org/10.1055/a-2044-0203>.
- [10] A. Wilson, V.A. Hodgetts-Morton, E.J. Marson, A.D. Markland, E. Larkai, A. Papadopoulou, et al., Tocolytics for delaying preterm birth: a network meta-analysis, *Cochrane Database Syst. Rev.* 8 (8) (2022), <https://doi.org/10.1002/14651858.CD014978.pub2>.
- [11] P. Stelzl, S. Kehl, P. Oppelt, H. Maul, S. Enengl, I. Kyvernitakis, W. Rath, Do obstetric units adhere to the evidence-based national guideline? A Germany-wide survey on the current practice of initial tocolysis, *Eur. J. Obstet. Gynecol. Reprod. Biol.* 270 (2022) 133–138, <https://doi.org/10.1016/j.ejogrb.2022.01.006>.
- [12] P. Stelzl, S. Kehl, W. Rath, Maintenance tocolysis: a reappraisal of clinical evidence, *Arch. Gynecol. Obstet.* 300 (5) (2019) 1189–1199, <https://doi.org/10.1007/s00404-019-05313-7>.
- [13] M.R. Peltier, Immunology of term and preterm labor, *RBE* 1 (1) (2003) 122, <https://doi.org/10.1186/1477-7827-1-122>.
- [14] A. Young, A.J. Thomson, M. Ledingham, F. Jordan, I.A. Greer, J.E. Norman, Immunolocalization of proinflammatory cytokines in myometrium, cervix, and fetal membranes during human parturition at term, *Biol. Reprod.* 66 (2) (2002) 445–449, <https://doi.org/10.1095/biolreprod66.2.445>.
- [15] Kim, S.H. MacIntyre DA, M.F. Da Silva, A.M. Blanks, Y.S. Lee, S. Thornton, et al., Oxytocin activates NF- $\kappa$ B-mediated inflammatory pathways in human gestational tissues, *Mol. Cell Endocrinol.* 403 (2015) 64–77, <https://doi.org/10.1016/j.mce.2014.11.008>.
- [16] S.H. Kim, L. Riaposova, H. Ahmed, O. Pohl, A. Chollet, J.-P. Gotteland, et al., Oxytocin receptor antagonists, atosiban and nolasiban, inhibit prostaglandin F $_{2\alpha}$ -induced contractions and inflammatory responses in human myometrium, *Sci. Rep.* 9 (1) (2019) 5792, <https://doi.org/10.1038/s41598-019-42181-2>.
- [17] L. Riaposova, S.H. Kim, A.C. Hanyaloglu, L. Sykes, D.A. MacIntyre, P.R. Bennett, V. Terzidou, Prostaglandin F $_{2\alpha}$  requires activation of calcium-dependent signalling to trigger inflammation in human myometrium, *Front Endocrinol.* 14 (2023) 1150125, <https://doi.org/10.3389/fendo.2023.1150125>.
- [18] R. Agoha, *Medicinal plants of Nigeria*, Faculteit der Wiskunde en Natuurwetenschappen, Nijmegen, Netherlands, 1973, p. 129.
- [19] A. Kamboj, A.K. Saluja, *Bryophyllum pinnatum* (Lam.) Kurz.: phytochemical and pharmacological profile: a review, *Phcog Rev.* 3 (6) (2009) 364.
- [20] C.A. Lans, Ethnomedicines used in Trinidad and Tobago for urinary problems and diabetes mellitus, *J. Ethnobiol. Ethnomed.* 2 (1) (2006) 45, <https://doi.org/10.1186/1746-4269-2-45>.
- [21] M. Hamburger, O. Potterat, K. Fürer, A.P. Simões-Wüst, U. von Mandach, *Bryophyllum pinnatum*-reverse engineering of an anthroposophic herbal medicine, *Nat. Prod. Commun.* 12 (8) (2017) 1359–1364, <https://doi.org/10.1177/1934578X1701200847>.
- [22] S. Santos, C. Haslinger, K. Klaic, M.T. Faleschini, M. Mennet, O. Potterat, et al., A bufadienolide-enriched fraction of *Bryophyllum pinnatum* inhibits human myometrial contractility *in vitro*, *Planta Med* (2018), <https://doi.org/10.1055/a-0810-7704>.
- [23] S. Santos, C. Haslinger, M. Mennet, U. von Mandach, M. Hamburger, A.P. Simões-Wüst, *Bryophyllum pinnatum* enhances the inhibitory effect of atosiban and nifedipine on human myometrial contractility: an *in vitro* study, *BMC Complement. Alter. Med* 19 (1) (2019) 1–12, <https://doi.org/10.1186/s12906-019-2711-5>.
- [24] B. Gwehenberger, L. Rist, R. Huch, U. von Mandach, Effect of *Bryophyllum pinnatum* versus fenoterol on uterine contractility, *Eur. J. Obstet. Gynecol. Reprod. Biol.* 113 (2) (2004) 164–171, [https://doi.org/10.1016/S0301-2115\(03\)00370-1](https://doi.org/10.1016/S0301-2115(03)00370-1).
- [25] A.P. Simões-Wüst, M. Grãos, C.B. Duarte, R. Brenneisen, M. Hamburger, M. Mennet, et al., Juice of *Bryophyllum pinnatum* (Lam.) inhibits oxytocin-induced increase of the intracellular calcium concentration in human myometrial cells, *Phytomedicine* 17 (12) (2010) 980–986, <https://doi.org/10.1016/j.phymed.2010.03.005>.
- [26] S. Santos, L. Zurlfluh, M. Mennet, O. Potterat, U. Von Mandach, M. Hamburger, A.P. Simões-Wüst, *Bryophyllum pinnatum* compounds inhibit oxytocin-induced signaling pathways in human myometrial cells, *Front Pharm.* 12 (2021) 142, <https://doi.org/10.3389/fphar.2021.632986>.
- [27] L. Zurlfluh, M. Giovannini Spinelli, C. Betschart, A.P. Simões-Wüst, Repurposing of *Bryophyllum pinnatum* for dysmenorrhea treatment: a systematic scoping review and case series, *Front Pharm.* 14 (2023), <https://doi.org/10.3389/fphar.2023.1292919>.
- [28] K. Fürer, M. Raith, R. Brenneisen, M. Mennet, A.P. Simões-Wüst, U. von Mandach, et al., Two new flavonol glycosides and a metabolite profile of *Bryophyllum pinnatum*, a phytotherapeutic used in obstetrics and gynaecology, *Planta Med* 79 (16) (2013) 1565–1571, <https://doi.org/10.1055/s-0033-1350808>.
- [29] S. Bachmann, C. Betschart, J. Gerber, K. Fürer, M. Mennet, M. Hamburger, et al., Potential of *Bryophyllum pinnatum* as a detrusor relaxant: an *in vitro* exploratory study, *Planta Med* 83 (16) (2017) 1274–1280, <https://doi.org/10.1055/s-0043-109097>.
- [30] K. Fürer, D. Eberli, C. Betschart, R. Brenneisen, M. De Mieri, M. Hamburger, et al., Inhibition of porcine detrusor contractility by the flavonoid fraction of *Bryophyllum pinnatum*—a potential phytotherapeutic drug for the treatment of the overactive bladder syndrome, *Int J. Phytomedicine* 22 (1) (2015) 158–164, <https://doi.org/10.1016/j.phymed.2014.11.009>.
- [31] M. Oufir, C. Seiler, M. Gerodetti, J. Gerber, K. Fürer, M. Mennet-von Eiff, et al., Quantification of bufadienolides in *Bryophyllum pinnatum* leaves and manufactured products by UHPLC-ESIMS/MS, *Planta Med* 81 (12/13) (2015) 1190–1197, <https://doi.org/10.1055/s-0035-1546126>.
- [32] D. Spiess, M. Winker, A. Dolder Behna, C. Gründemann, A.P. Simões-Wüst, Advanced *in vitro* safety assessment of herbal medicines for the treatment of non-psychotic mental disorders in pregnancy, *Front Pharm.* 13 (2022) 882997, <https://doi.org/10.3389/fphar.2022.882997>.
- [33] R. Wächter, R. Brenneisen, M. Hamburger, M. Mennet, M. Schnelle, A. Worel, et al., Leaf press juice from *Bryophyllum pinnatum* (Lam) Oken induces myometrial relaxation, *Phytomedicine* 19 (1) (2011) 74–82, <https://doi.org/10.1016/j.phymed.2011.06.032>.
- [34] X. Zhou, S.W. Seto, D. Chang, H. Kiat, V. Razmovski-Naumovski, K. Chan, A. Bensoussan, Synergistic effects of Chinese herbal medicine: a comprehensive review of methodology and current research, *Front Pharm.* 7 (2016) 201, <https://doi.org/10.1016/j.fphar.2016.00201>.
- [35] L. Zurlfluh, L. Duvaud, N. Inci, O. Potterat, A.P. Simões-Wüst, J. Mosbacher, *Bryophyllum pinnatum* inhibits oxytocin and vasopressin signaling in myometrial cells, *Planta Med* (2024), <https://doi.org/10.1055/a-2303-9608>.
- [36] S. Arrowsmith, Oxytocin and vasopressin signalling and myometrial contraction, *Curr. Opin. Physiol.* 13 (2020) 62–70, <https://doi.org/10.1016/j.cophys.2019.10.006>.
- [37] S.H. Kim, P.R. Bennett, V. Terzidou, Advances in the role of oxytocin receptors in human parturition, *Mol. Cell Endocrinol.* 449 (2017) 56–63, <https://doi.org/10.1016/j.mce.2017.01.034>.
- [38] S. Iacovides, I. Avidon, F.C. Baker, What we know about primary dysmenorrhea today: a critical review, *Hum. Reprod. Update* 21 (6) (2015) 762–778, <https://doi.org/10.1093/humupd/dmv039>.
- [39] V. Lundström, K. Green, Endogenous levels of prostaglandin F $_{2\alpha}$  and its main metabolites in plasma and endometrium of normal and dysmenorrheic women, *Am. J. Obstet. Gynecol.* 130 (6) (1978) 640–646, [https://doi.org/10.1016/0002-9378\(78\)90320-4](https://doi.org/10.1016/0002-9378(78)90320-4).
- [40] K. Fürer, A.P. Simões-Wüst, U. von Mandach, M. Hamburger, O. Potterat, *Bryophyllum pinnatum* and related species used in anthroposophic medicine:

- constituents, pharmacological activities, and clinical efficacy, *Planta Med* 82 (11-12) (2016) 930–941, <https://doi.org/10.1055/s-0042-106727>.
- [41] A.P. Simões-Wüst, O. Lapaire, I. Hösl, R. Wächter, K. Fürer, M. Schnelle, et al., Two randomised clinical trials on the use of *Bryophyllum pinnatum* in preterm labour: results after early discontinuation, *Complement Med Res* 25 (4) (2018) 269–273, <https://doi.org/10.1159/000487431>.
- [42] K. Fürer, A.P. Simões-Wüst, A. Winkler, N. Amsler, M. Schnelle, U. von Mandach, The application of *Bryophyllum pinnatum* preparations in obstetrics and gynaecology - a multicenter, prospective observational study. *Forschende Komplementärmedizin* 22 (4) (2015) 231–236, <https://doi.org/10.1159/000437154>.
- [43] N.B. Meirowitz, J.C. Smulian, R.A. Hahn, P. Zhou, S. Shen-Schwarz, G.H. Lambert, et al., Collagen messenger RNA expression in the human amniochorion in premature rupture of membranes, *Am. J. Obstet. Gynecol.* 187 (6) (2002) 1679–1685, <https://doi.org/10.1067/mob.2002.127595>.
- [44] J. Vink, V. Yu, S. Dahal, J. Lohner, C. Stern-Asher, M. Mourad, et al., Extracellular matrix rigidity modulates human cervical smooth muscle contractility—new insights into premature cervical failure and spontaneous preterm birth, *Reprod. Sci.* 28 (1) (2021) 237–251, <https://doi.org/10.1007/s43032-020-00268-6>.
- [45] U. Puistola, L. Risteli, A. Kauppila, M. Knip, J. Risteli, Markers of type I and type III collagen synthesis in serum as indicators of tissue growth during pregnancy, *J. Clin. Endocrinol. Metab.* 77 (1) (1993) 178–182, <https://doi.org/10.1210/jcem.77.1.8325940>.
- [46] A. Ouellette, M. Mahendroo, S. Nallasamy, Collagen and elastic fiber remodeling in the pregnant mouse myometrium, *Biol. Reprod.* 107 (3) (2022) 741–751, <https://doi.org/10.1093/biolre/iaoc102>.
- [47] O. Shynlova, P. Tsui, S. Jaffer, S.J. Lye, Integration of endocrine and mechanical signals in the regulation of myometrial functions during pregnancy and labour, *Eur. J. Obstet. Gynecol. Reprod. Biol.* 144 (2009) S2–S10.
- [48] E. Zehorai, T. Gross Lev, E. Shimshoni, R. Hadas, I. Adir, O. Golani, et al., Enhancing uterine receptivity for embryo implantation through controlled collagenase intervention, *Life Sci. Alliance* 7 (10) (2024) e202402656, <https://doi.org/10.26508/lsa.202402656>.
- [49] S.S. Mathew-Steiner, S. Roy, C.K. Sen, Collagen in Wound Healing, *Bioengineering* 8 (5) (2021) 63, <https://doi.org/10.3390/bioengineering8050063>.
- [50] G. Gantner, D. Spiess, E. Randecker, K.C. Quack Lötscher, A.P. Simões-Wüst, Use of herbal medicines for the treatment of mild mental disorders and/or symptoms during pregnancy: a cross-sectional survey, *Front Pharm.* 12 (2021) 729724, <https://doi.org/10.3389/fphar.2021.729724>.
- [51] N. Plangger, L. Rist, R. Zimmermann, U. von Mandach, Intravenous tocolysis with *Bryophyllum pinnatum* is better tolerated than  $\beta$ -agonist application, *Eur. J. Obstet. Gynecol. Reprod. Biol.* 124 (2) (2006) 168–172, <https://doi.org/10.1016/j.ejogrb.2005.05.013>.
- [52] A.R.A. McDougall, R. Hastie, M. Goldstein, A. Tuttle, A. Ammerdorffer, A. M. Gülmezoglu, J.P. Vogel, New medicines for spontaneous preterm birth prevention and preterm labour management: landscape analysis of the medicine development pipeline, *BMC Pregnancy Childbirth* 23 (1) (2023) 525, <https://doi.org/10.1186/s12884-023-05842-9>.
- [53] C. Betschart, U. von Mandach, B. Seifert, D. Scheiner, D. Perucchini, D. Fink, V. Geissbühler, Randomized, double-blind placebo-controlled trial with *Bryophyllum pinnatum* versus placebo for the treatment of overactive bladder in postmenopausal women, *Phytomedicine* 20 (3-4) (2013) 351–358, <https://doi.org/10.1016/j.phymed.2012.10.007>.
- [54] T. Huber, D. Krüerke, T. Haack, M. Weber, M. Kröz, M. Schlemmer, A.P. Simões-Wüst, Perceived Changes in Anxiety Symptom Burden during Treatment with *Bryophyllum pinnatum*: A Prospective, Single-Arm Study, *Pharmaceuticals* 17 (11) (2024) 1423, <https://doi.org/10.3390/ph17111423>.
- [55] C. Lambrigger-Steiner, A.P. Simões-Wüst, A. Kuck, K. Fürer, M. Hamburger, U. Von Mandach, Sleep quality in pregnancy during treatment with *Bryophyllum pinnatum*: an observational study, *J. Phytomed* 21 (5) (2014) 753–757, <https://doi.org/10.1016/j.phymed.2013.11.003>.
- [56] N. Mirzayeva, S. Forst, D. Passweg, V. Geissbühler, A.P. Simões-Wüst, C. Betschart, *Bryophyllum pinnatum* and improvement of nocturia and sleep quality in women: a multicentre, nonrandomised prospective trial, *Evid. Based Complement. Alter. Med* (2023), <https://doi.org/10.1155/2023/2115335>.
- [57] A.P. Simões-Wüst, T.A. Hassani, B. Müller-Hübenthal, S. Pittl, A. Kuck, H. Meden, et al., Sleep quality improves during treatment with *Bryophyllum pinnatum*: an observational study on cancer patients, *ICT* 14 (5) (2015) 452–459, <https://doi.org/10.1177/1534735415580680>.
- [58] J. Kolodziejczyk-Czepas, A. Stochmal, Bufadienolides of *Kalanchoe* species: an overview of chemical structure, biological activity and prospects for pharmacological use, *Phytochem. Rev.* 16 (6) (2017) 1155–1171, <https://doi.org/10.1007/s11101-017-9525-1>.
- [59] Y. Liu, Y. Luo, M. Li, Z. Li, J. Lan, L. Lu, et al., Comparative study on pharmacokinetics of extract from *Bufo venenum* and its liposomes, *J. Pharm. Biomed. Anal.* 249 (2024) 116375, <https://doi.org/10.1016/j.jpba.2024.116375>.
- [60] I.M. Usta, A. Khalil, A.H. Nassar, Oxytocin antagonists for the management of preterm birth: a review, *Am. J. Perinatol.* 28 (6) (2011) 449–460, <https://doi.org/10.1055/s-0030-1270111>.
- [61] A. Cassidy, A.M. Minihane, The role of metabolism (and the microbiome) in defining the clinical efficacy of dietary flavonoids, *Am. J. Clin. Nutr.* 105 (1) (2017) 10–22, <https://doi.org/10.3945/ajcn.116.136051>.

## Glossary

*Tocolysis*: pharmaceutical therapy aiming at inhibiting preterm myometrial contractions to prevent preterm birth