Pharmaceutical research on *Bryophyllum pinnatum* – *in vitro* effects on human myometrial contractility

Inauguraldissertation
zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel
von

Stefanie Lopes dos Santos

2020

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

auf Antrag von

PD Dr. Ana Paula Simões-Wüst

Prof. Dr. Matthias Hamburger

Prof. Dr. Muriel Cuendet

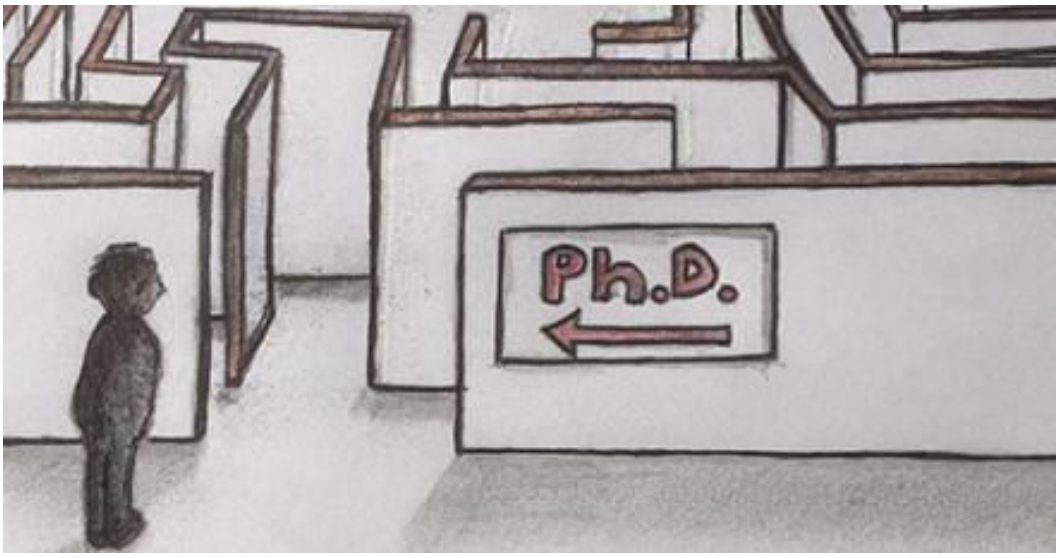
Basel, den 15.09.2020

Prof. Dr. Martin Spiess

Dekan

‘Simplicity is the ultimate sophistication’

Leonardo da Vinci (1452-1519)



Sketch by PhD students from the University of York

Abstract

Bryophyllum pinnatum is a succulent perennial plant used in the treatment of premature labour, first in anthroposophic medicine and, recently in many perinatal clinics in Switzerland, as a monotherapy or add-on therapy with known tocolytic agents. The good effectiveness, as well as the very good tolerability, have been confirmed by several studies. Earlier experimental evidence obtained from i*n vitro* studies supports its use. Preterm birth is the number one cause of newborn mortality and morbidity, and often results from preterm labour. Since preterm uterine contractions are frequently associated with preterm birth, their inhibition by tocolytics may delay delivery long enough (24-48 h) to achieve foetal lung maturation. Currently used tocolytics include oxytocin (OT) receptor antagonists (e.g. atosiban), calcium channel blockers (e.g. nifedipine) and β-sympathomimetics, among others. Due to tocolytics' side effects and insufficient therapeutic effects, additional therapeutic options are needed. For the administration of a phytotherapeutic, it is necessary to have knowledge not only about their therapeutic effect and toxicity, but also about their mode of action. This thesis describes the effects of *B. pinnatum* on human myometrium contractility.

Because a combination of different drugs might prove to be helpful in achieving a prolongation of pregnancy in more patients, *B. pinnatum* in combination with the two tocolytics was tested *in* vitro. Myometrium strips placed under tension in an organ bath were allowed to contract spontaneously. The addition of *B. pinnatum* press juice (BPJ), atosiban, and nifedipine moderately reduced the strength (area under the curve (AUC) and amplitude) of contractions. When BPJ was added together with atosiban or nifedipine, the reduction of contraction strength was significantly higher than with the tocolytics alone. The inhibitory effects of BPJ plus atosiban and of BPJ plus nifedipine on contractions strength were concentration-dependent and, none of the substances, alone or in combination, decreased myometrial cell viability.

A previous metabolite profile study of *B. pinnatum* leaves showed that flavonoid glycosides and bufadienolides are the major classes of secondary metabolites. Fractions enriched in flavonoid glycosides (FEF) and bufadienolides (BEF) were therefore prepared, and their effects on human myometrial contractility were characterised. The repeated addition of FEF, flavonoid aglycon mixture (A-Mix), BEF, or BPJ to the spontaneously contracting human myometrium, led to a

progressive decrease of contraction strength, without jeopardising the vitality of the myometrium strips. None of the compounds decreased myometrial cell viability, even at higher concentrations than those used in the myometrium experiments. Results suggest that bufadienolides might be important for the inhibition of myometrium contractility.

Finally, the effects of BPJ compounds on myometrium contractility were studied at the cellular level. The inhibitory effects of BPJ, BEF, FEF, A-Mix, bersaldegenin-1,3,5-orthoacetate (BO), the combination of BEF plus FEF and BEF plus A-Mix on the secondary intracellular effects triggered by OT, such as changes in intracellular calcium levels and phosphorylation of mitogen activated protein kinases (MAPKs) were compared. BPJ led to a concentration-dependent decrease of the OT-induced increase of intracellular calcium concentration ($[Ca^{2+}]_i$) in two myometrium cell lines, achieving ca. 75% inhibition. BEF, FEF, A-Mix, BO, and both combinations led to a concentration-dependent decrease of the OT-induced increase of $[Ca^{2+}]_i$ in hTERT-C3 cells. BEF, FEF, BO, and A-Mix, at concentrations corresponding to BPJ, led to a ca. 25% decrease of the OT-induced increase of $[Ca^{2+}]_i$. The combination of BEF plus FEF led to a decrease of 55.3% while BEF plus A-Mix led to a decrease of 38%. In addition, BPJ significantly reduced OT-induced phosphorylation of MAPKs SAPK/JNK and ERK1/2 at its maximum (5 min incubation). Also, at the cellular level, the results suggest that bufadienolides might be mainly responsible for the inhibitory effect.

The insights gained from the intensive inhibition of human myometrium contractility by *B. pinnatum* are promising and support its use as an add-on medication with therapeutic potential.

Zusammenfassung

Bryophyllum pinnatum ist eine sukkulente, mehrjährige Pflanze, welche zur Behandlung frühzeitiger Wehen eingesetzt werden kann. Zunächst in der Anthroposophischen Medizin angewandt findet die Pflanze heutzutage in vielen Geburtskliniken in der Schweiz als Alleintherapie sowie in Kombination mit anderen bekannten Wehenhemmern Verwendung. Ihre gute Wirksamkeit sowie die ausgezeichnete Verträglichkeit konnten von mehreren Studien bestätigt werden. Bisherige Resultate aus *in vitro*-Studien unterstützen deren Gebrauch. Frühgeburt ist die Hauptursache für Mortalität und Morbidität und wird oft durch verfrühtes Eintreten der Wehen hervorgerufen. Da frühzeitige Uteruskontraktionen oftmals mit Frühgeburt assoziiert sind, kann deren Unterdrückung durch Wehenhemmer den Gebärprozess lange genug (24-48 h) hinauszögern, um die fötale Lungenreifung zu erreichen. Zurzeit angewandte Wehenhemmer sind unter anderem. Oxytocin (OT)-Rezeptorantagonisten (z.B. Atosiban), Kalziumkanalblocker (z.B. Nifedipin) und β-Sympathomimetika. Aufgrund vorhandener Nebenwirkungen sowie ungenügender therapeutischer Wirksamkeit besteht Bedarf an zusätzlichen therapeutischen Optionen. Für die Verabreichung eines Phytotherapeutikums ist es nötig, Kenntnisse über dessen therapeutischen Wirksamkeit, Toxizität sowie auch dessen Wirkmechanismus zu erlangen. Die vorliegende Arbeit beschreibt Effekte von *B. pinnatum* auf die Kontraktilität humanen Myometriums.

Eine Kombination verschiedener Medikamente könnte für eine Verlängerung der Schwangerschaft in mehr Patientinnen hilfreich sein. Unter Spannung gesetzte Myometrium-Streifen wurden in ein Organbad gelegt und deren Spontankontraktionen wurden analysiert. Das Zugeben von *B. pinnatum*-Presssaft (BPJ), Atosiban und Nifedipin führten zu einer mässig reduzieren Stärke der Kontraktionen (Fläche unter der Kurve (AUC) und Amplitude). Bei Zugabe von BPJ zusammen mit Atosiban oder Nifedipin, war die Reduktion der Kontraktionsstärke signifikant höher als mit den beiden Wehenhemmern allein. Die inhibitorischen Effekte von BPJ mit Atosiban und BPJ mit Nifedipin auf die Kontraktionsstärke waren konzentrationsabhängig und keine der Substanzen, ob allein oder in Kombination, führte zu einer verringerten myometrialen Zellviabilität.

Eine frühere Studie zum Metabolitenprofil von *B. pinnatum*-Blättern zeigte, dass Flavonoidglykoside und Bufadienolide die Hauptklassen von Sekundärmetaboliten sind. Es

wurden Flavonoidglykosid- und Bufadienolid-angereicherte Fraktionen (FEF und BEF) hergestellt und deren Effekte auf die Kontraktilität von humanem Myometrium wurde charakterisiert. Wiederholte Zugabe von FEF, Flavonoidaglykonmixtur (A-Mix), BEF oder BPJ zum spontan kontrahierenden humanen Myometrium führte zu einer progressiven Abnahme der Kontraktionsstärke, ohne die Vitalität der Myometriumsstreifen zu beeinträchtigen.

Keine der Wirkstoffe führte zu einer verminderten myometrialen Zellviabilität, nicht einmal bei höheren Konzentrationen als in den Myometriumsstreifen-Kontraktionsexperimenten verwendet. Diese Resultate suggerieren, dass Bufadienolide für die Inhibition der Myometriumkontraktilität von Wichtigkeit sind.

Letztlich wurden die Effekte von BPJ-Wirkstoffen auf die Myometriumkontraktilität auf der Zellebene studiert. Die hemmenden Effekte von BPJ, BEF, FEF, A-Mix, Bersaldegenin-1,3,5-Orthoacetat (BO), der Kombination von BEF und FEF sowie BEF und A-Mix auf die sekundären intrazellulären durch OT hervorgerufenen Effekte, wie z.B. Änderungen der intrazellulären Kalziumwerte ($[Ca^{2+}]_i$) und der Phosphorylierung von Mitogen-aktivierten Proteinkinasen (MAPKs) wurden verglichen. BPJ führte zu einer konzentrationsabhängigen Senkung der OT-induzierten Steigerung der $[Ca^{2+}]_i$ in zwei myometrialen Zelllinien und erreichte ca. 75% Inhibition. BEF, FEF, A-Mix, BO und beide Kombinationen führten zu einer konzentrationsabhängigen Senkung der OT-induzierten $[Ca^{2+}]_i$ in hTERT-C3-Zellen. BEF, FEF, BO und A-Mix führten zu einer a. 25%igen Senkung des OT-induzierten Anstiegs der $[Ca^{2+}]_i$ bei Konzentrationen, die denen im BPJ entsprechen. Die Kombination von BEF und FEF führte zu einer Senkung von 55.3%, während BEF und A-Mix zu einer Senkung von 38% führten. Zudem verringerte BPJ die OT-induzierte Phosphorylierung von den MAPKs SAPK/JNK und ERK1/2 an deren Maximum (5 min Inkubation) signifikant. Überdies suggerieren Resultate auf der Zellebene, dass Bufadienolide hauptverantwortlich für den hemmenden Effekt sind.

Die aus der intensiven Hemmung von humaner Myometriumkontraktilität durch *B. pinnatum* gewonnen Erkenntnisse sind vielversprechend und bekräftigen dessen Verwendung als Ergänzungsmedikament mit therapeutischem Potential.

Resumo

Bryophyllum pinnatum é uma planta perene suculenta utilizada no tratamento de trabalho de parto prematuro, primeiro na medicina antroposófica e, mais recentemente em muitas clínicas perinatais na Suíça, como monoterapia ou terapia complementar de agentes tocolíticos. Uma boa eficácia, bem como a sua ótima tolerância, foi confirmada por vários estudos. Experiências *in vitro* apoiam a sua utilização. O nascimento prematuro é a primeira causa de mortalidade e morbilidade de recém-nascidos e resulta maioritariamente do trabalho de parto prematuro. Dado que as contrações uterinas estão frequentemente relacionadas com o parto prematuro, a sua inibição por tocolíticos pode atrasar o parto o tempo suficiente (24-48 h) para permitir a maturação pulmonar do feto. Os tocolíticos utilizados atualmente incluem antagonistas dos recetores de ocitocina (OT) (*e.g.* atosiban), bloqueadores dos canais de cálcio (*e.g.* nifedipina) e β-simpatomiméticos, entre outros. Devido aos efeitos secundários dos tocolíticos e à insuficiência de efeitos terapêuticos, são necessárias opções terapêuticas adicionais. Para a administração de medicamentos fitoterapêuticos é necessário ter conhecimentos sobre o seu efeito terapêutico e toxicidade, mas também, sobre o seu modo de ação. Esta tese descreve efeitos de *B. pinnatum* na contractilidade do miométrio humano.

A combinação de drogas pode ajudar a conseguir um prolongamento da gravidez num número maior de pacientes. Tiras de miométrio colocadas sob tensão num banho de órgãos foram deixadas a contrair espontaneamente. A adição de sumo de *B. pinnatum* (BPJ), atosiban, e nifedipina reduziu moderadamente a força (área sob a curva (AUC) e a amplitude) das contrações. Quando BPJ foi adicionado em combinação com atosiban ou nifedipina, a redução da força de contração foi significativamente mais elevada do que apenas com os tocolíticos. Os efeitos inibidores de BPJ mais atosiban e de BPJ mais nifedipina na força de contração são dependentes da concentração e, nenhuma das substâncias, isolada ou em combinação, diminuiu a viabilidade das células miometriais.

Um estudo anterior do perfil metabólico das folhas de *B. pinnatum* mostrou que os flavonoides glicosídeos e os bufadienolideos são as principais classes de metabolitos secundários presentes. Foram preparadas frações enriquecidas em glicosídeos flavonoides (FEF) e bufadienolides (BEF), e os seus efeitos na contractilidade do miométrio humano foram caracterizados. A adição repetida de FEF, uma mistura de flavonoides agliconas (A-Mix), BEF,

ou BPJ, ao miométrio humano em contração espontânea, levou a uma diminuição progressiva da força da contração, sem comprometer a vitalidade das tiras de miométrio. Nenhum dos compostos diminuiu a viabilidade das células miometriais, mesmo em concentrações mais elevadas do que as utilizadas nas experiências *in vitro* de miométrio. Os resultados sugerem que os bufadienolideos são importantes para a inibição da contração do miométrio.

Finalmente, o efeito dos compostos do BPJ na contração do miométrio foi estudado a nível celular. Foram comparados os efeitos inibidores do BPJ, BEF, FEF, A-Mix, bersaldegenin-1,3,5-ortoacetato (BO), a combinação de BEF com FEF e BEF com A-Mix, nos efeitos intracelulares secundários desencadeados pela OT, tais como alterações nos níveis intracelulares de cálcio e fosforilação das proteínas-quinases ativadas por mitógeno (MAPK). BPJ levou a uma diminuição, concentração-dependente, do aumento da concentração intracelular de cálcio ($[Ca^{2+}]_i$) induzido pela OT em duas linhas celulares de miométrio, alcançando uma inibição de cerca de 75%. BEF, FEF, A-Mix, BO, e ambas as combinações levaram a uma diminuição dependente da concentração do aumento da $[Ca^{2+}]_i$ induzido pela OT nas células hTERT-C3. BEF, FEF, BO e a A-Mix, em concentrações correspondentes a BPJ, levaram a uma diminuição da $[Ca^{2+}]_i$ induzido por OT de cerca de 25%. A combinação de BEF mais FEF levou a uma diminuição de 55,3% enquanto que BEF mais A-Mix levou a uma diminuição de 38%. Além disso, BPJ reduziu significativamente a fosforilação induzida por OT das MAPK SAPK/JNK e ERK1/2 no seu máximo (5 minutos de incubação). Os resultados sugerem que os bufadienolideos podem ser os principais responsáveis pelo efeito inibidor.

Os conhecimentos obtidos a partir da inibição intensiva da contração do miométrio humano por *B. pinnatum* são promissores e apoiam o seu uso como medicamento adicional com potencial terapêutico.

Acknowledgements

Nobody said it would be easy. This was only possible due to the passion, enthusiasm and work of a tone of amazing people. I could have never done this alone. This thesis belongs to each and every one of the people who in one way or another contributed, knowing or maybe without even knowing. Thank you! Danke schön! Obrigada!

I would like to thank my thesis committee members for evaluating this thesis and the work involved. Thank you to **Prof. Dr. Muriel Cuendet** and my PhD supervisors, **PD Dr. Ana Paula Simões-Wüst** and **Prof. Dr. Matthias Hamburger**. I am looking forward to our discussions.

First and foremost, I am specially and profoundly thankful to **Paula** for her insatiable commitment to this project, for her continuous encouragement and for believing in me. In the past four years a lot of memories, good moments, and a lot of knowledge were accumulated. Thank you, Paula! I know how much you have given of yourself in each step of this big project, and I want to thank you deeply. It was a pleasure to be your first PhD student, and I can only wish that our pathways will keep on crossing.

Prof. Hamburger thank you for agreeing to be my doctoral father. It has been a privilege to work under your supervision, and I am grateful for your support and trust in me during my time as a doctoral student. Thank you for sharing your knowledge, experience, and expertise with me.

Warm thanks to **Dr. Monica Mennet**, **Dr. Martin Schnelle**, **Prof. Dr. Olivier Potterat**, **Prof. Dr. Ursula von Mandach**, and all the **Bryophyllum Study Group** for the pleasant and fruitful collaboration. Thank you for all the comments and ideas during all the meetings we had together over the past four years.

I would also like to thank **Dr. sc. nat. Martin Ehrbar** for the opportunity to 'belong' to the Ehrbar's Lab and participating in the joint lab meetings, which were always very interesting and have brought me closer to new research fields. Thank you also for all the discussions and inputs.

Further thanks go to **Prof. Dr. med. Roland Zimmermann** and the **clinic of obstetrics** of the University Hospital of Zurich, who made the realization of the study possible. I am especially

thankful to **Christian Haslinger** for his insatiable commitment to this project, for all the hard work in collecting all the myometrium tissue, and also for the good discussions.

I acknowledge everyone who helped somewhere along the way. Thank you **Mario Grãos** for all the help with the calcium protocol, and **Prof. Aguzzi** for allowing me to use your facilities. While working on this project I was allowed to meet so many people – **Mark**, **Dezirae**, **Lorene**, **Emma** – you guys were amazing.

An enormous big thank you to you **Alex**! I shared an exceptional journey with you. I greatly appreciate your help, patience, competence, optimism and friendship. You were a continuingly inspiring person to me, and I will treasure the considerable advice you have passed on to me. Thank you a thousand times, simply for everything! Thank you **Kathi** for all the help, all the problems solved in the western blot life ('Blot baby'), I know you will miss me singing for you! Of course, this work also belongs to **Kristian**, **Leonie**, and **Fabienne** that were the best students I could have asked for. Thank you for all the moments; you have also taught me a lot during your stay in the lab. **Lisa** for the amazing breakfast break talks and brain juices, I am already missing them. **Debbie**, the lab was not the same without you, working as a team is power. Special thanks to **Yvonne** and **Corina** for your warm-heartedness, and your kindness. I will deeply miss our daily greeting ceremonies ('Hallo'). **Esther**, **Queralt**, **Eva**, **Yannick**, and **Sybille** for all the conversations, coffee breaks and, of course, all the help. Thank you all for all the good moments from the past four years that I will never forget. For all the laughs, coffees, and ice creams. For all the dancing parties in the cell lab that nobody saw. It has been a delight and a great honour to work with all of you. Thank you for the most wonderful and unforgettable time!

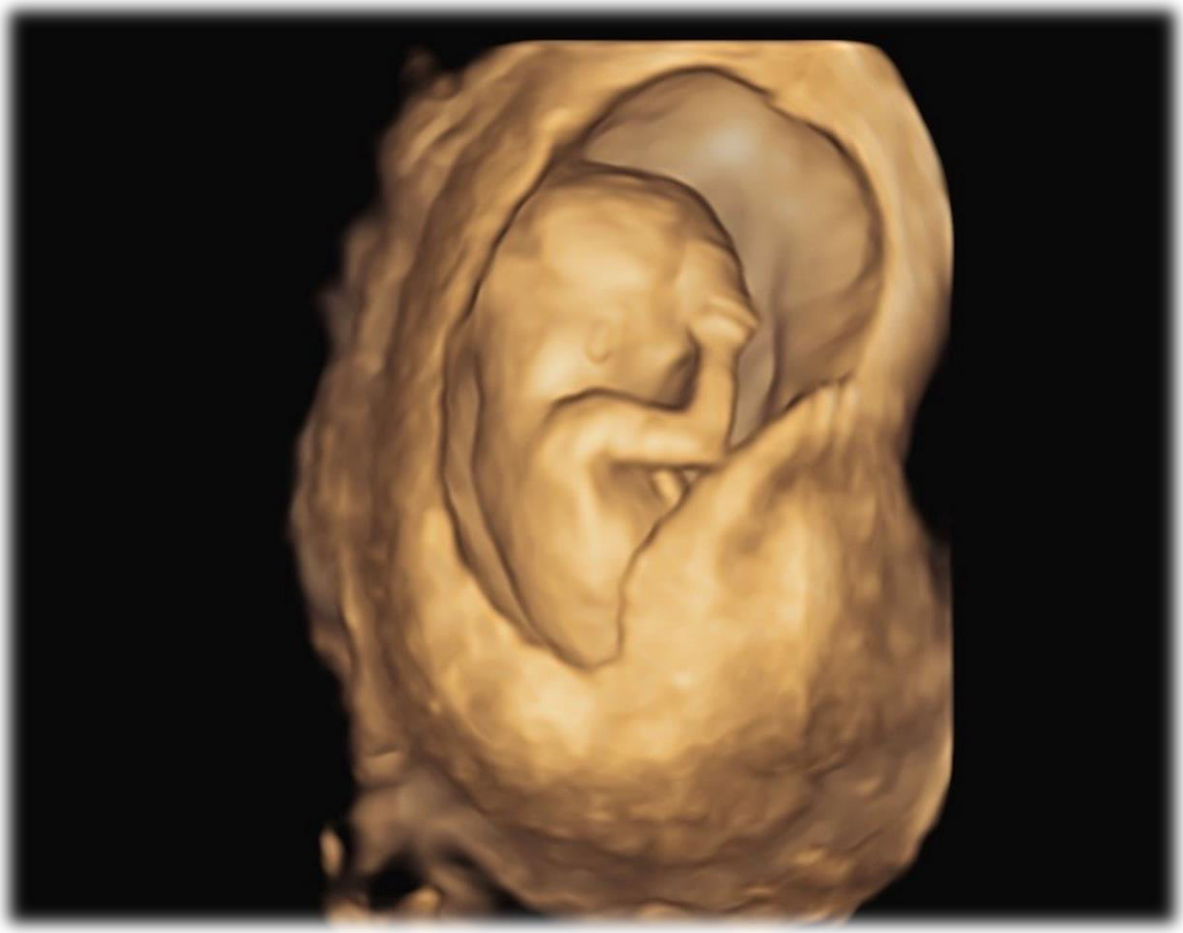
I would also like to thank all the members of the **pharmaceutical biology group** at the University of Basel, but especially **Teresa**, **Jakob**, **Amy**, **Lara**, **Ombeline**, and **Nova** for receiving me as one of your own even when we would only see each other a few times a year.

Very special and heartfelt thanks to my dear **family and friends**! For your encouragements, understanding and belief in me. Knowing that you stand by my side has empowered me to achieve what I have. You are a blessing for my life! Thank you, **Bruno,** for all the patience, love, friendship, and strength that you gave me during this journey. I cannot wait for what is coming next! Thank you and love you! Thank you, **Daniela**, **Ricardo**, **Susana**, **Bruno G.**, **Sofia**, **Márcia**,

Daniel, **Marilia**, and **Filipe** for all the good moments, all the dinners, laughs and conversations over the past years. Thank you for the friendship!

Thank you **Ana Luzio** for all the words of motivation and for listening to me in the hardest moments. Your strength and positivity kept me going to arrive to this moment. Your help along the way was amazing. Thank you!

Thank you all who are not mentioned here, but who in some way have contributed to this work.



Personal photo of baby at 12 weeks of pregnancy, 27th April 2020.

List of abbreviations

AGC	Antenatal glucocorticoids
AM	Anthroposophic Medicine
A-Mix	Flavonoid aglycon mixture
AP-1	Activator protein 1
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AUC	Area under the curve
BEF	Bufadienolide enriched fraction
BMI	Body mass index
BO	Bersaldegenin-1,3,5-orthoacetate
BPJ	*Bryophyllum pinnatum* leaf press juice
Ca^{2+}	Calcium ions
Ca^{2+}-CaM	Ca^{2+}-calmodulin complex
$[Ca^{2+}]_i$	Intracellular calcium concentration
CaM	Calmodulin
CCB	Calcium channel blocker
CH_2Cl_2	Dichloromethane
CNS	Central nervous system
COX	Cyclooxygenase
CPI-17	C-kinase-activated protein phosphatase-1 inhibitor 17 kDa
$cPLA_2$	Cytosolic phospholipase A_2
CRH	Corticotropin-releasing hormone
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
EBV-EA	Epstein-Barr virus early antigen
EvaMed	Evaluation of anthroposophic medicine
ERK	Extracellular-signal regulated kinase
FEF	Flavonoid enriched fraction
GABA	γ-aminobutyric acid
GDI	GDP-dissociation inhibitor

GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
G-protein	Guanylate nucleotide binding protein
GTP	Guanosine triphosphate
GTPases	Guanosine triphosphatases
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
H_2O	Water
ICS	International continence society
IP_3	Inositol 1,4,5-triphosphate
IP_3R	Inositol-tris-phosphate specific receptor
i.v.	Intravenous
MAPK	Mitogen-activated protein kinase
MeOH	Methanol
MHC	Myosin heavy chain
MKK	MAPK kinase
MKKK	MAPK kinase kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MUI	Urgency-dominant mixed urinary incontinence
MYPT1	Myosin phosphatase target subunit 1
NICU	Neonatal intensive care unit
NO	Nitric oxide
OAB	Overactive bladder syndrome
OT	Oxytocin
OTR	Oxytocin receptor
PG	Prostaglandin
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGH_2	Prostaglandin H_2
PGHS	PGH_2 endoperoxide synthase-2

PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC-β	Phospholipase C-β
PM	Plasma membrane
p.o.	'per os' (oral administration)
PP1	Protein phosphatase-1
PPROM	Prelabour premature rupture of membranes
PTB	Preterm birth
PTGS	Prostaglandin endoperoxide synthase
PTL	Preterm labour
RDS	Respiratory distress syndrome
RhoA	Ras homolog family member A
ROCK	RhoA-associated kinase
SAPK/JNK	Stress-activated protein kinase or c-Jun N-terminal
s.c.	Subcutaneous
SOCE	Store-operated calcium entry
SOCs	Store operated channels
Swissmedic	Swiss Agency for therapeutic products
SR	Sarcoplasmic reticulum
UV	Ultra violet
VGCC	Voltage-gated Ca^{2+} channel

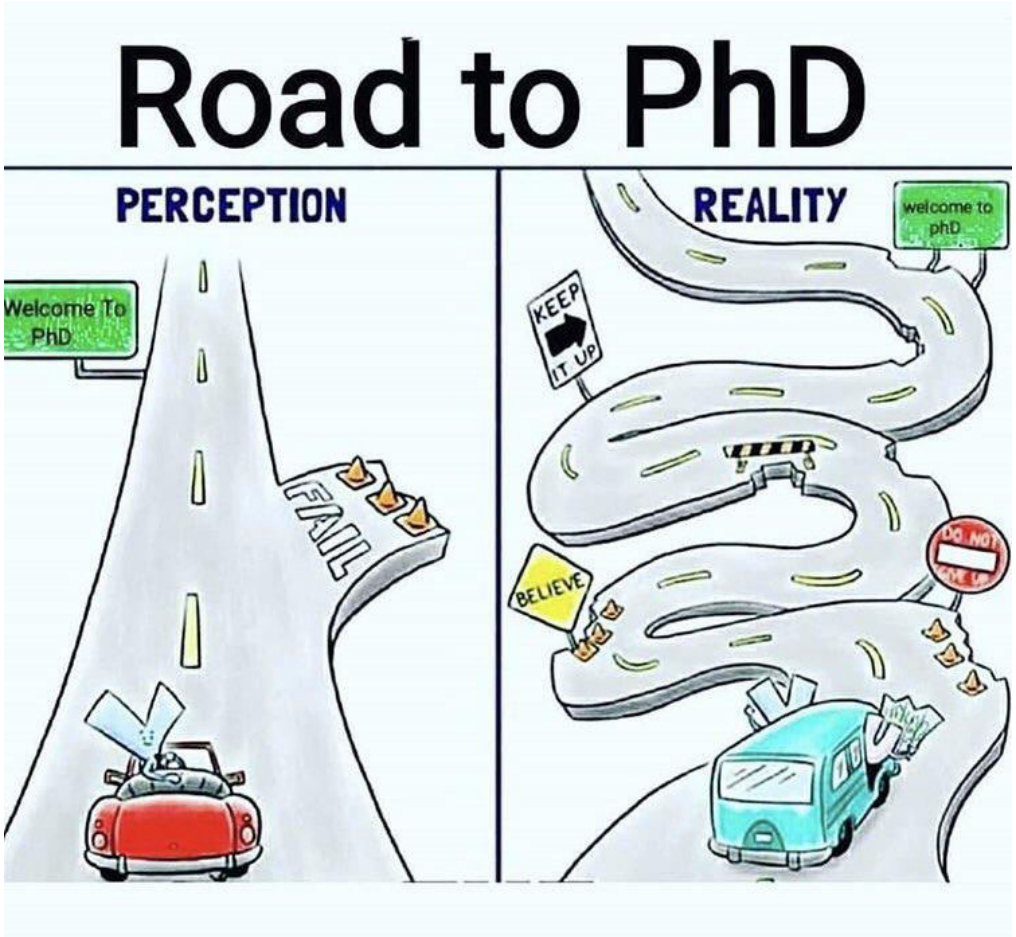
Table of Contents

Abstract VII
Zusammenfassung IX
Resumo XI
Acknowledgements XIII
List of abbreviations XVII

SCOPE OF THE THESIS 3
Motivation 5
Specific aims 6
Thesis outline 6
CHAPTER 1 9
Introduction
1.1 Bryophyllum pinnatum 11
1.1.1 History 12
1.1.2 Phytochemistry 12
1.1.3 Anthroposophic medicine 17
1.1.4 Pharmacological and clinical activities 18
1.1.4.1 Tocolysis 19
1.1.4.2 Overactive bladder syndrome 20
1.1.4.3 Sleep disorders 21
1.1.4.4 Other indications 21
1.1.4.5 Tolerability and toxicity 22
1.2 Human uterus 23
1.2.1 Uterus anatomy 23
1.2.2 Smooth muscle contraction 24
1.3 Preterm labour 27
1.3.1 Pathophysiology 27
1.3.2 Risks and causes 28
1.3.3 Management of preterm labour 29

1.3.3.1 Foetal lung maturation 29
1.3.3.2 Tocolytics 30
1.4 Contractility pathways 32
1.4.1 Role of oxytocin 32
1.4.2 Oxytocin receptor 34
1.4.3 Main oxytocin-induced signalling pathways 35
1.4.3.1 Canonical pathway 35
1.4.3.2 Calcium sensitization - RhoA/ROCK pathway 37
1.4.3.2 MAPK pathway 38
1.5 References 41
CHAPTER 2 49
Myometrium contractility
2.1. *Bryophyllum pinnatum* enhances the inhibitory effect of atosiban and nifedipine on human myometrial contractility: an *in vitro* study 51
2.2 A bufadienolide-enriched fraction of *Bryophyllum pinnatum* inhibits human myometrial contractility 65
CHAPTER 3 83
Oxytocin induced pathways
3.1 *Bryophyllum pinnatum* compounds inhibit oxytocin-induced signalling pathways in human myometrial cells 85
CHAPTER 4 117
Conclusions and outlook
4.1 Closing remarks 119
4.2 General discussion 120
4.3 Future directions 124
4.4 References 127

Scope of the thesis


Road to PhD
PERCEPTION
Welcome To PhD
FAIL
REALITY
welcome to phD
KEEP IT UP
BELIEVE

Motivation

During pregnancy, mother and baby are in a vulnerable state. Since health issues or illnesses of the mother need to be treated and pharmacological therapies can potentially be harmful to the unborn child, safe alternatives should be considered. A pregnancy complication that can have dramatic consequences is preterm labour (PTL). Preterm is defined as birth before 37 weeks of pregnancy, affects 5 to 18% of pregnancies, and is the number one cause of mortality and morbidity in newborns. PTL can be caused by many different processes, and treatments used in clinics address the symptoms more than the underlying cause. Since the most commonly recognized symptom is the contracting uterus, most treatments focus on relaxing the uterus (tocolysis). The inhibition of uterus contractions by tocolytics may delay delivery long enough (24-48h) to achieve foetal lung maturation and *in uterus* transfer to a unit care facility. So far, no maintenance or long-term tocolytic treatments have proved to be effective. Due to tocolytics' insufficient therapeutic efficacy as well as their numerous and, in part, serious side-effects, additional therapeutic options are needed.

Bryophyllum pinnatum, a succulent perennial plant, was introduced in the 1970s as an alternative tocolytic agent by anthroposophic medicine in Europe. Today, *B. pinnatum* preparations are being used in many perinatal clinics in Switzerland, as a monotherapy or add-on therapy in cases of PTL. Its good effectiveness, as well as its very good tolerability, could have been confirmed in several studies. Earlier *in vitro* experiments performed with human myometrium strips revealed that *B. pinnatum* preparations reduce contractions' strength. Furthermore, experiments with myometrium cells have shown that *B. pinnatum* leaf press juice (BPJ) can prevent the increase of the intracellular calcium concentration ($[Ca^{2+}]_i$) induced by oxytocin (OT), a hormone known to trigger myometrium contractions.

Aware of the current limitations of tocolytic treatments and with the long-term goal of reducing the prevalence of preterm birth (PTB), we have explored various *in vitro* models to gain further knowledge about the mechanism of action of *B. pinnatum* compounds.

Specific aims

The overall aim of this project is to study the therapeutic effect of *B. pinnatum* on human myometrial contractility *in vitro* but also, toxicity and mode of action.

To achieve the outlined research goal, this thesis has the following specific aims:

1. **To evaluate the effects of *B. pinnatum* in combination with atosiban and with nifedipine on human myometrium contractility.** Chewable tablets of *Bryophyllum pinnatum* are often used in clinical practice as an add-on medication. However, we do not know if the combination with known tocolytic drugs brings an advantage in lowering the strength of myometrial contractility.
2. **To find out which are the *B. pinnatum* compounds that lead to a reduction of human myometrium contractility.** The presence of several compounds in *B. pinnatum* was previously shown. In order to better determine which compound might be responsible for *B. pinnatum*'s effect on human myometrium contractility, we propose to compare the effect of previously prepared fractions/compounds with the inhibitory effect of *B. pinnatum* in human myometrium contractility.
3. **To gain more information on the OT-induced signalling pathways that are inhibited by *B. pinnatum* compounds**. The effect of *B. pinnatum* leaf press juice on the $[Ca^{2+}]_i$ was previously shown. However, we do not know which compound might be responsible for promoting this effect. Also, the information regarding the effect of *B. pinnatum* on other OT-induced signalling pathways is still unexplored. For this reason, we propose to expand knowledge on the effect of *B. pinnatum* on the MAPKs pathway, as well as that of previously prepared fractions/compounds.

Thesis outline

This thesis contains four chapters that, taken together, support the use of *B. pinnatum* preparations as a treatment for PTL. In **Chapter 1**, the history of the therapeutic use and of previous studies on *B. pinnatum* preparations are discussed. Only after understanding how

difficult the clinical treatment of PTL is and how the contractility of myometrium is dependent on the activation of several pathways, will we be able to evaluate how *B. pinnatum* can interfere with the underlying processes.

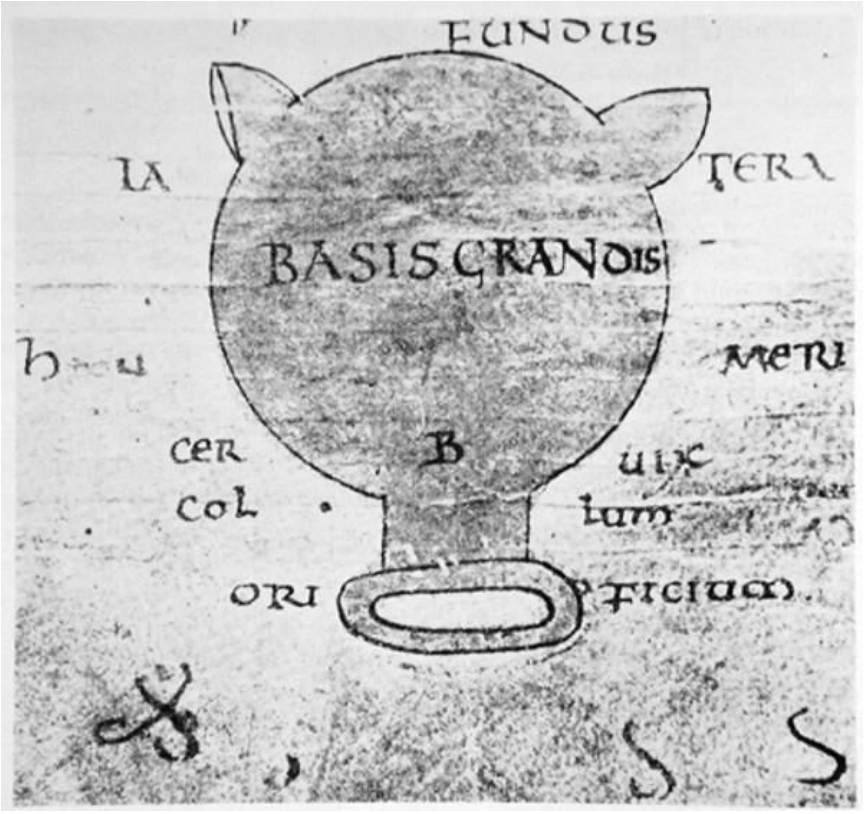
The combination of different drugs might be needed to achieve a stronger inhibition of myometrium contractility and prolong pregnancy in a higher number of patients. To investigate this, as presented in **Chapter 2**, we decided to obtain myometrium biopsies from women undergoing a caesarean section, and place strips from this tissue under tension in an organ bath (myograph). Strips were allowed to contract spontaneously, and the effects of the combinations BPJ plus atosiban and BPJ plus nifedipine on contractions were studied. This effect was analysed by studying the strength (area under the curve (AUC) and amplitude) as well as frequency of contractions. Motivated by the discoveries, in the second part of this chapter we studied the effect of the major classes of secondary metabolites of *B. pinnatum* leaves in the same set up. Fractions enriched in flavonoid glycosides (FEF) and in bufadienolides (BEF) were prepared, and their effects on human myometrial contractility were characterised. FEF, a flavonoid aglycon mixture (A-Mix), BEF, or BPJ, were repeatedly added to the spontaneously contracting human myometrium.

Next, eager to understand the mechanism of action behind these findings, we decided to study the effect of BPJ fractions/compounds on the OT-induced signalling pathways. In **Chapter 3** the inhibitory effects of BPJ, BEF, FEF, A-Mix, bersaldegenin-1,3,5-orthoacetate (BO), the combinations BEF plus FEF and BEF plus A-Mix, and the OT-receptor antagonist atosiban were studied on the secondary intracellular effects triggered by OT, namely on changes in intracellular calcium levels and phosphorylation of mitogen activated protein kinases (MAPKs).

Taken together, the main messages obtained following the various approaches are discussed in **Chapter 4**, which also comprises a section on future perspectives. We believe that this thesis provides a solid foundation for future research on *B. pinnatum*, its components and its use in the treatment of PTL.

Chapter 1

Introduction



Earliest surviving illustration of uterine anatomy (9th century)

1.1 *Bryophyllum pinnatum*

Bryophyllum pinnatum (Lamarck) Oken (syn. *Kalanchoe pinnata* (Lamarck) Persoon), syn.: *Bryophyllum calycinum* (Salisbury) (Fig. 1A) belongs to the family of Crassulaceae and is known by numerous vernacular names, such as cathedral bells, life plant, air plant, love plant, miracle leaf, and Goethe plant [1]. *B. pinnatum* is a perennial succulent plant that originated in Madagascar, and now grows widely across tropical regions of Africa, America, India, China, and Australia [2]. The name *Bryophyllum* is based on the Greek words 'bryon' which means 'sprout' or 'grow' and 'phyllon' which translates to 'leaf'.

Figure 1. *B. pinnatum* plant, plantlet and blooming.
B. pinnatum grows to about 1-1.5 m tall and the leaves are succulent and fleshy dark green **(A)**. The plant has a special mode of reproduction, whereby little plantlets sprout at the edges of the leaves. When the leaf falls to the ground, the new plantlets grow roots and develop into a fully grown plant **(B)**. The *B. pinnatum* plant develops pendulous flowers **(C)** [3-5].

This herbaceous plant has a fleshy, cylindrical stem and grows to a height of about 1-1.5 m. The decussate arranged leaves are succulent and fleshy dark green. At the base of the stem the leaves are simple, and at the top they are imparipinnate, 10-30 cm long, with three to five pairs of fleshy limb lobes.

B. pinnatum plants have a rather unique mode of vegetative reproduction, whereby young plantlets develop on the edges of the leaves when they fall to the ground (Fig 1B).

B. pinnatum develops gorgeous inflorescences from November to March, and fruits in April. The pendulous flowers are coloured violet on top, then fade from green to reddish and consist of a tubular and inflated calyx of 2-4 cm (Fig. 1C) [2].

1.1.1 History

In 1783, *Cotylet pinné* was described for the first time by Jean-Baptiste Lamarck (1744-1829) in the "Encyclopédie méthodique". After the first specimen was imported from India (Calcutta) to England, Christian Hendrik Persoon (1755-1837) reclassified it in the Kalanchoe, calling it *Calanchoe pinnata* (with an orthographic variant). Twenty years later, in 1805, Richard Anthony Salisbury (1762-1829) wrote the first description of *Bryophyllum calycinum*, creating the new genus *Bryophyllum* [6].

During the Continental Blockade (1806-1814), the exchange of knowledge was stopped and the plant was described independently amongst scientists. In 1907, Raymond-Hamet formally introduced the species from genus *Bryophyllum* into genus *Kalanchoe* [7]. In 1948/49, Pierre Boiteau and Octave Mannoni merged the genus *Bryophyllum* and *Kitchingia* with *Kalanchoe* and gave them the ranking of sections according to the rules of nomenclature [8]. On the other hand, in 1930, Alwin Berger and August Theodor Harms kept the genus *Bryophyllum* independent [9].

Johann Wolfgang von Goethe (1749-1832) was first acquainted with *Bryophyllum calycinum* in the Botanical Garden (Vienna, Austria) and recorded his observations in his diary for the first time in 1818. He began to investigate the plant extensively, and occupied himself with it until his death. Years after Goethe's death, his manuscripts and studies were summarized and published by Georg Balzer in his book "Goethe's *Bryophyllum*. Ein Beitrag zu seiner Pflanzenmorphologie".

1.1.2 Phytochemistry

The known secondary metabolites present in *B. pinnatum* include bufadienolides, flavonoids, alkaloids, various phenolics, triterpenes, steroids, lipids, fatty acids, minerals, and vitamins [2, 10]. Phytochemical studies have focused mostly on bufadienolides, which are cardiotoxic thus leading to a greater interest amongst researchers, and flavonoids, because they are rather abundant.

Bufadienolides

Bufadienolides are a new type of natural steroid. Its derivatives include many in the form of bufadienolide glycosides (bufadienolides that contain structural groups derived from sugars). The term bufadienolides originated from 'bufo', a genus of toads which contain large amounts of bufadienolide glycosides in their venom, the suffix -adien-, which refers to the two double bonds in the lactone ring, and the ending -olide, which denotes the lactone structure [11, 12].

These are important cardiac glycosides that increase the contractile force of the heart by inhibiting the enzyme Na^+/K^+-ATPase [12]. They have many bioactive properties such as sedative, insecticidal, cytotoxic, antitumor, positive inotropic and cardiotonic [13-16]. Due to their distribution among the plant organs, it is believed that the main role of bufadienolides is chemical plant protection [17].

Eight bufadienolides (Nr. 1-8) have been identified from leaves, flowers, stems and roots of *B. pinnatum* (Fig. 2). A characteristic structural feature is the 1,3,5-orthaoacteate function that is present in some of the compounds. The bufadienolides identified were: bersaldegenin-1-acetate (1), bersaldegenin-3-acetate (2), bryotoxin C (equal to bryophyllin A, 3), bryophyllin C (4), bersaldegenin-1,3,5-orthoactetate (BO, 5), bryophyllin B (6), bryotoxin B (7), and bryotoxin A (8) [10].

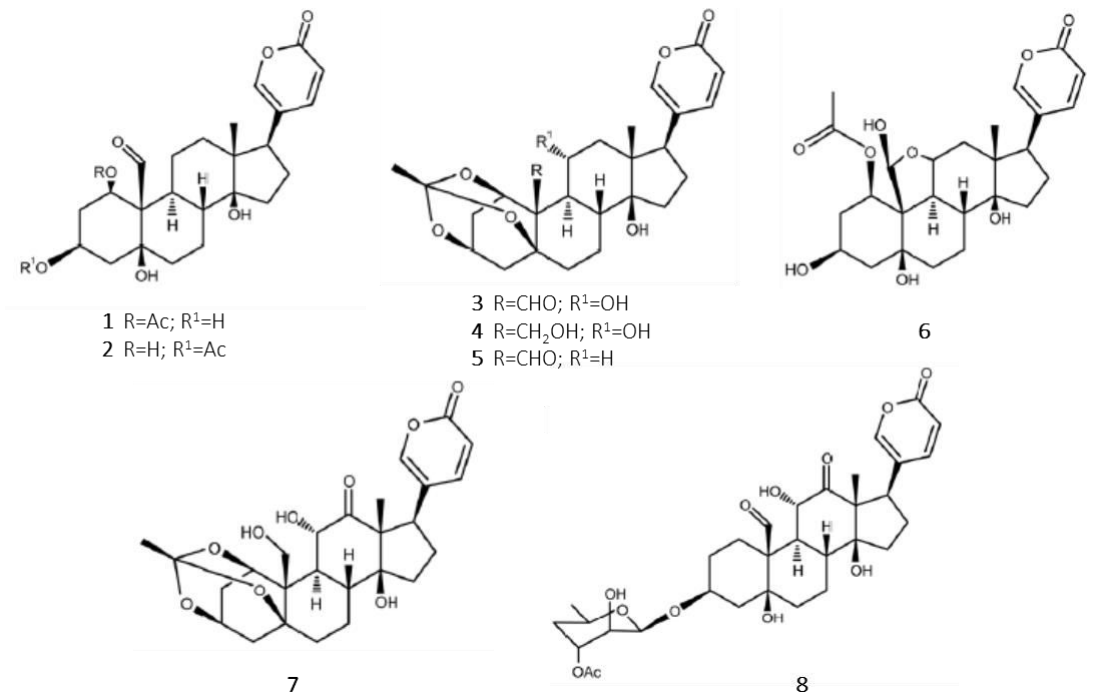


Figure 2. Bufadienolides reported in *B. pinnatum*.
A total of eight bufadienolides have been reported in *B. pinnatum*. Figure adapted from Fürer K. *et al.*, 2016 [17].

Flavonoids

Flavonoids are plant pigments that are synthesised from phenylalanine. They generally display marvellous colours known from flower petals, mostly emiting brilliant fluorescence when excited by ultra violet (UV) light, and are ubiquitous to green plant cells [18]. They are commonly found in fruit, vegetables, nuts, seeds, flowers, tea, wine, and honey [19, 20].

The function of flavonoids is to regulate plant growth hormones and growth regulators, as well as the induction of gene expression [19, 20]. Flavonoids are becoming the subject of medical research due to their many useful properties, including enzyme inhibition, such as reverse transcriptase and protease, and antimicrobial, anti-inflammatory [21], antiallergic [19], antioxidant and cytotoxic activity [22]. In addition, their toxicity to animals is low [23].

Flavonoids are the main metabolites of *B. pinnatum*, and include numerous flavonol derivates (Nr. 9-30) with kaempferol and quercetin glycosides being the most important flavonoid representatives, as well as some flavonoles (Nr. 31-35). The flavonoids identified were (Fig. 3): kaempferol (9), kapinnatoside (kaempferol 3-O-α-L-arabinopyranosyl-(1 → 2)-α-L-

rhamnopyranoside, 10), kaempferol 3-O-β-D-xylopyranosyl-(1 → 2)- α-L-rhamnopyranoside (11), kaempferitrin (kaempferol 3-O,7-O-di-α-L-rhamnopyranoside, 12), kaempferol 3-O-α-L-(2-O-acetyl) rhamnopyranoside 7-O-α-L-rhamnopyranoside (13), kaempferol 3-O-α-L-(3-O-acetyl) rhamnopyranoside 7-O-α-L-rhamnopyranoside (14), kaempferol 3-O-α-L-(4-O-acetyl) rhamnopyranoside 7-O-α-L-rhamnopyranoside (15), kaempferol 3-O-α-D-glucopyranoside 7-O-α-L-rhamnopyranoside (16), Afzelin (kaempferol 3-O-α-L-rhamnopyranoside, 17), α-rhamnoisorobin (kaempferol 7-O-α-L-rhamnopyranoside, 18), astragalin (kaempferol 3-O-β-D-glucopyranoside, 19), myricitrin (myricetin 3-O-α-L-rhamnopyranoside, 20), myricetin 3-O-α-L-arabinopyranosyl-(1 → 2)-α-L-rhamnopyranoside (21), quercetin (22), 3’,4’-Di-O-methylquercetin (23), quercetin 3-O-α-L- arabinopyranosyl-(1 → 2)-α-L-rhamnopyranoside (24), quercitrin (quercetin 3-O-α-L-rhamnopyranoside, 25), quercetin 3-O-α-L-arabinopyranosyl-(1 → 2)-α-L-rhamnopyranoside 7-O-β-D-glucopyranoside (26), isoquercitrin (quercetin 3-O-β-D-glucopyranoside, 27), miquelianin (quercetin 3-O-β-D-glucuronopyranoside, 28), rutin (quercetin 3-O-rutinoside, 29), 3,5,7,3’,5’-pentahydroxyflavone (30), luteolin (31), luteolin 7-O-β-D-glucopyranoside (32), diosmine (diosmetin 7-O-α-L- rhamnopyranosyl-(1 → 6)-β-D- glucopyranoside, 33), acacetin 7-O-α-L-rhamnopyranosyl-(1 → 6)-β-D- glucopyranoside (34), and 4’,5-7-trihydroxy-3’,8-dimethoxyflavone 7-O-β-D-glucopyranoside (35) [10].

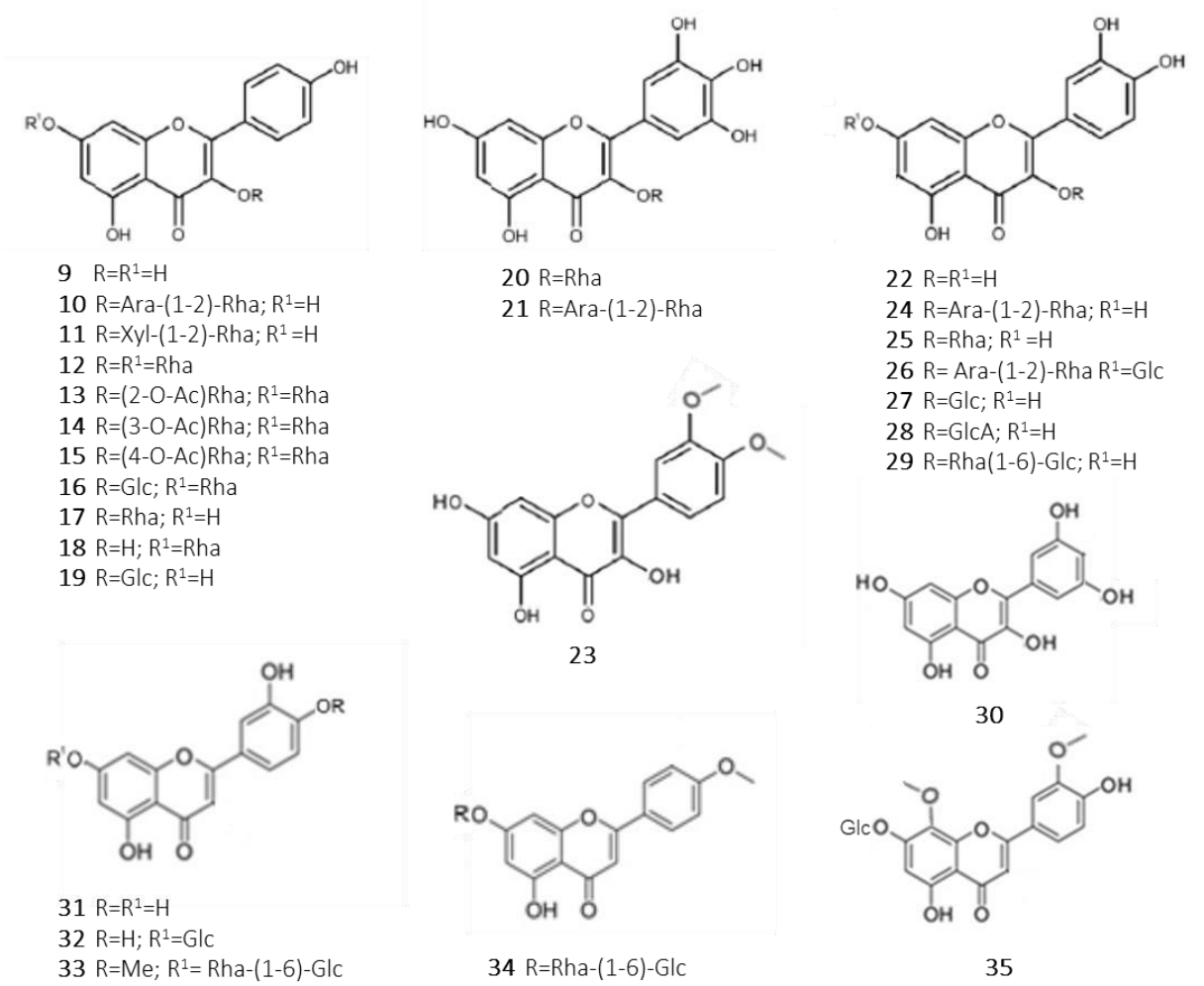


Figure 3. Flavonoids reported in *B. pinnatum*.
A total of 27 flavonoids have been reported in *B. pinnatum*. Figure adapted from Fürer K. *et al.*, 2016 [17].

B. pinnatum fractionation

To facilitate the investigation of the biological effects of bufadienolides and flavonoids, fractions enriched in these compounds were prepared [24, 25]. Leaves of *B. pinnatum* cultivated in Schwäbisch Gmünd, Germany (greenhouse) were harvested and kept frozen until processed. Frozen leaves were lyophilised and powdered in a mortar, and the leaf powder was extracted with methanol (MeOH) and evaporated. A portion of the MeOH extract was partitioned between dichloromethane (CH_2Cl_2) and water (H_2O). The aqueous phase was fractionated by column chromatography to provide a fraction enriched in flavonoids (FEF) and a polar fraction, containing sugars and L-malic acid. Evaporation of the CH_2Cl_2 phase yielded a bufadienolide fraction [24]. The bufadienolides fraction obtained was further purified by solid-

phase extraction to obtain a fraction enriched in bufadienolides (BEF; Fig. 4) [25]. These fractions were used in the experimental work described in chapters 2 and 3.

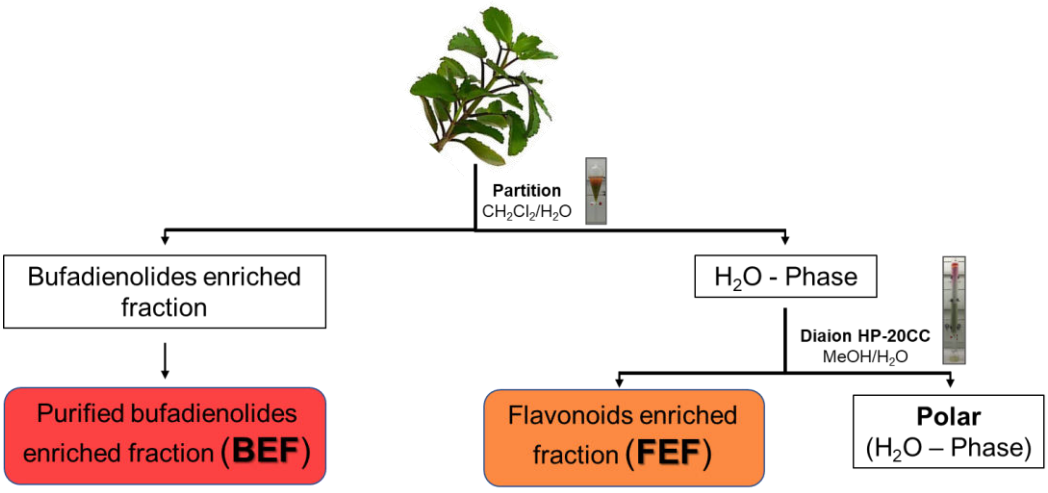


Figure 4. Schematic representation of *B. pinnatum* leaf fractionation
Leaves of *B. pinnatum* (from Schwäbisch Gmünd, Germany) were lyophilised and powdered in a mortar, and the powder was extracted with MeOH. After evaporation, a portion of the MeOH extract was partitioned between CH_2Cl_2 and H_2O. The aqueous phase was fractionated by column chromatography on Diaion HP-20 to provide a fraction enriched in flavonoids (FEF) and a polar fraction. Evaporation of the CH_2Cl_2 phase yielded a bufadienolide fraction [24] that was further purified by solid-phase extraction (BEF) [25].

1.1.3 Anthroposophic medicine

Anthroposophic medicine (AM) is an integrative multimodal medical system that was established by the Austrian philosopher Rudolf Steiner (1861-1925). After studying empirical sciences, mathematics, and philosophy in Vienna, at the age of 22 Steiner was commissioned to publish Johann Wolfgang Goethe's scientific writings in *Kürschners Deutscher Nationalliteratur* (German National Literature) [26]. Steiner began developing anthroposophy in 1901 [27].

In 1921, the first anthroposophic hospital was established in Arlesheim, Switzerland, by Dr. Ita Wegman (1876-1943). AM is practiced by physicians fully trained and qualified in university medicine. The main principle involves integrating conventional skills and methods with a holistic understanding of man and nature and of disease and treatment. From this point of

view, the understanding of the human being in his/her entirety means acceptance of a three-system organisation with a physical body, a soul, and a spirit. The holistic view of the human being leads to an understanding of health and illness that differs from that of conventional medicine and to treatments that are specifically adapted to each individual [28]. Anthroposophy is a view of humanity and nature that is spiritual but that at the same time regards itself to be profoundly scientific.

In Europe, the use of remedies prepared from *B. pinnatum* leaves were recommended in 1921 by Rudolf Steiner as anthroposophic medicines to treat hysteria [17, 29]. Hysteria was described as a condition in which all spiritual and emotional energy is poured into an action and, therefore, the body is no longer capable of regulating normal physical reactions [30]. In 1970, Dr. Werner Hassauer introduced *B. pinnatum* as a routine treatment to prevent premature labour (PTL) in AM hospitals. From an AM point of view, an imbalance in the astral (e.g. emotions) and etheric (physiological) organisations can lead to cramps, vaginal infections and, therefore, to PTL. The health interaction is supported by *B. pinnatum* [31].

1.1.4 Pharmacological and clinical activities

Various preparations of *B. pinnatum* are currently available commercially. A multi-centre observational study performed by 38 German physicians in collaboration with the Evaluation of Anthroposophic Medicine (EvaMed) network showed that for over six years, a total of 4038 prescriptions were recorded in the EvaMed data bank in a broad range of therapeutic indications [32].

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 medical product without any indication. *B. pinnatum* products are available in many formulations. The main commercial products used include “Bryophyllum 50% powder”: leaf press juice adsorbed to lactose; “Bryophyllum chewable tablets 350 mg 50%”: leaf press juice adsorbed to lactose; “Bryophyllum Dilutio 33%: ethanolic leaf extract, for oral application; and “Bryophyllum ampoules 5%”: aqueous leaf extract, for subcutaneous (s.c.) and intravenous (i.v.) administration.

In Switzerland, *B. pinnatum* preparations are used for the treatment of premature contractions, anxiety, restlessness, and sleep disorders [33, 34]. *In vitro* and *in vivo* research activities that have been performed are described in the following sections in chronological order.

1.1.4.1 Tocolysis

In 1970, the German gynaecologist, Dr. Hassauer, introduced *B. pinnatum* as a tocolytic agent when he was looking for a suitable alternative to the commonly used tocolytic fenoterol, since the latter was causing a great deal of adverse side effects in the treated women. His study showed that treatment with *B. pinnatum* 5% infusion (i.v.) and 50% trituration (oral administration ('per os': p.o.)) was well tolerated and successful in 84% of women, and results were comparable to conventional treatments. Treatment with *B. pinnatum* also enabled the dosage of conventional treatments to be reduced or even replaced [31]. These results were supported by two subsequent studies, where *B. pinnatum* showed a comparable positive outcome to fenoterol and no side effects were registered [35]. In addition, the incidence of premature deliveries decreased to 1.07% [36].

Some years ago, in order to collect more clinical, pharmacological and chemical information regarding the use of *B. pinnatum* as a herbal medicine, an interdisciplinary team of scientists joined forces in the *Bryophyllum Study Group*. In 2003, the first *in vitro* study on the effect of *B. pinnatum* on myometrial contractility was performed. The relaxing effect of *B. pinnatum* aqueous leaf was compared with that of the β-agonist fenoterol on contracting human myometrium strips obtained from biopsies taken from women undergoing term caesarean section. This study confirmed the relaxant effect of *B. pinnatum* and supported further research [37]. In 2005, a retrospective matched-pair study involving 67 pregnant women compared the tolerability and tocolytic activity of *B. pinnatum* 5% i.v. with the β-agonists fenoterol and hexoprenaline. The study showed similar maternal and neonatal outcomes in both treatment groups. However, in the group receiving *B. pinnatum*, maternal adverse effects (palpitation, dyspnoea) were significantly reduced, and the use of corticosteroids and antibiotics was lower [38].

In a different set-up, the mechanism behind the tocolytic effect of *B. pinnatum* was investigated using human myometrial cells. Leaf pressed juice of *B. pinnatum* (BPJ) led to a concentration-

dependent inhibition of the oxytocin (OT)-induced increase of the intracellular calcium concentration ($[Ca^{2+}]_i$) and provided further support for the use of *B. pinnatum* as a tocolytic [39]. Next, BPJ and three fractions of *B. pinnatum* methanolic leaf extract were tested on spontaneous contractions in human myometrial strips. BPJ and a flavonoid enriched fraction (undiluted) significantly decreased the area under the curve (AUC) and amplitude of contractions. A rapid and large increase in frequency was observed with all substances tested [40].

In Switzerland, *B. pinnatum* is not only used in anthroposophically oriented clinics but also in main perinatal centres [33, 34]. At the University Hospital of Zurich, internal guidelines suggest the use of *B. pinnatum* preparations as a co-medication [34].

1 .1.4.2 Overactive bladder syndrome

Overactive bladder syndrome (OAB) is, as defined by the International Continence Society (ICS), a urinary urgency, with or without incontinence, related to high frequency and nocturia without obvious pathological causes, such as urinary tract infections [41].

The effect of BPJ on porcine detrusor muscle contractility was investigated in an organ bath chamber, with oxybutynin as a reference drug. The pressed juice (5% in the chamber) significantly inhibited detrusor contractility by 74.6% compared to control. In addition, 10% BPJ had a significant relaxant effect on carbachol-induced contractions. The leaf press juice showed good activity, although oxybutynin inhibitory and relaxant properties were more pronounced [42]. In a prospective, randomized, double-blind, placebo-controlled study, 20 postmenopausal women suffering from OAB or urgency-dominant mixed urinary incontinence (MUI) were treated with *B. pinnatum* 50% chewable tablets or placebo. After 8 weeks of treatment, a positive trend for the effect of *B. pinnatum* on micturition frequency/24 h could be shown compared to placebo [43].

Additional *in vitro* studies have investigated the effect of BPJ and different fractions of *B. pinnatum* on electrically induced porcine detrusor contractility [24, 25]. The inhibitory effect of BPJ was confirmed, even though an initial stimulatory effect was observed. FEF reduced muscle contractility in a dose- and time-dependent manner [24]. Treatment with BEF and flavonoid

aglycon mixture (A-Mix) led to a concentration-dependent lowering of the contraction force [25], supporting insights provided by preceding clinical studies.

1.1.4.3 Sleep disorders

In a prospective, multi-centre observational study, 49 pregnant women suffering from sleep disorders were treated with *B. pinnatum* 50% chewable tablets. After 14-day treatment, results showed that the quality of sleep had significantly improved, and the women felt less sleepy during the day [44]. An additional observational study revealed improvements in sleep quality of cancer patients after treatment with the same tablets [45].

1.1.4.4 Other indications

B. pinnatum is a well-regarded plant with a high phytotherapeutic potential. The leaves are particularly promising for the treatment of various disorders. Besides the applications mentioned, it also finds use in other indications [46].

Neurological disorders

Neuropharmacological studies of the *B. pinnatum* leaf extract were conducted in rats and mice. The results demonstrated that the methanolic fraction of *B. pinnatum* possesses a potent central nervous system (CNS) depressant action. The leaf extract significantly increased brain γ-aminobutyric acid (GABA) content in mice. GABA is known as an inhibitory neurotransmitter in a number of CNS pathways [47]. Furthermore, a *B. pinnatum* aqueous leaf extract also produced a significant neurosedative, CNS depressant, and anxiolytic effects in mice [48, 49].

Anticancer and antitumor activity

The effect of bufadienolides against several tumour cells was tested in an *in vitro* assay with human lung carcinoma A-549 cells, KB cells, and colon HCT-8 tumour cells. Bryophyllin A showed a potent cytotoxicity in all cell lines, Bersaldegenin-3-acetate showed an effect against HCT-8 cells, and Bryophyllin B mainly demonstrated an effect against KB cells [50].

An *in vitro* study showed that five bufadienolides possess anti-tumour promoting activity by inhibiting Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells. This investigation showed that the 1,3,5-orthoacetate moiety was important for the chemoprotective activity [51]. A few years later, an *in vitro* study using a *B. pinnatum* chloroform extract and a fraction containing steroidal glycosides, alkaloids, and steroids demonstrated a dose-dependent inhibition of human cervical cancer cell growth. The fraction was more potent than the extract for pro-apoptotic activity. On the other hand, the extract had higher anti-human papilloma virus (HPV) activity than the fraction [52].

Anti-inflammatory activity

The anti-inflammatory activity of *B pinnatum* aqueous leaf extract was studied in the paw oedema of Wistar rats, and a significant decrease was observed [53]. Another study confirmed this result, revealing a significant reduction of acute inflammation by the aqueous extract and a steroidal derivative, stigmas-4,20(21),23-trien-3-one [54]. Furthermore, the topical application of an ethanolic extract of *B. pinnatum* significantly reduced ear oedema in Swiss albino mice [55]. More recent work showed that differently dried extracts of *B. pinnatum* possess anti-inflammatory properties via the stabilization of human red blood cells membrane, inhibition of heat-induced haemolysis, and albumin denaturation. The different extracts also protected human erythrocytes against lipid peroxidation and increased levels of reduced glutathione, known markers of cellular degradation [56].

1.1.4.5 Tolerability and toxicity

B. pinnatum may be an excellent therapy option due to its good tolerability. Specifically if used as a tocolytic, it has shown significantly fewer side effects than other treatments such as betamimetics, which often cause palpitations and dyspnoea [38]. In addition, the treatment of 14 pregnant women with *B. pinnatum* chewable tablets demonstrated no side effects assigned to the medication [57].

A randomized, double-blind, placebo-controlled study on OAB showed that *B. pinnatum* preparations led to fewer side effects than other substances used for the treatment of OAB, which often go along with anticholinergic side effects [43]. In a multicentre prospective

observational study that considered sleep disorders and OAB as indications for *B. pinnatum*, tolerability was rated very high, and only a few adverse reactions were documented [33].

The toxicity of other *Bryophyllum* species based on the content of bufadienolides has been reported, and was observed with animals grazing on *B. pinnatum, B. tubiflorum*, and *B. daigremontianum* [16]. Cardiotoxicity of bufadienolides was investigated *in vitro* on isolated rabbit and guinea pig hearts, and a strong positive inotropic effect from two orthoacetates (bersaldegenin 1,3,5- orthoacetate (BO) and daigremontianin) was observed [14, 58]. On the other hand, toxicity was observed in mice and rats at very high doses [47, 59].

1.2 Human uterus

The growth and development of a new human being in a woman's womb is one of the most fascinating processes the human body is capable of. Pregnancy, also known as gestation, is an experience full of growth, change, enrichment, and challenge. It is the time during which one or more babies develop inside a woman, a process that typically occurs in approximately 40 weeks from the start of the last menstrual period (around 14 days before the fertilisation of the oocyte). The uterus is the most important organ in the reproductive process. Its importance starts with the nesting of the fertilized egg, then carrying of the embryo and ,later, the foetus, providing the perfect environment for its growth and development [60, 61].

1.2.1 Uterus anatomy

The uterus, or womb, is a major female hormone-responsive secondary sex organ of the reproductive system in humans and most mammals. It is located within the pelvic region immediately behind and almost overlying the bladder, and in front of the sigmoid colon. This human organ is pear-shaped and about 7.6 cm long, 4.5 cm broad and 3.0 cm thick [61]. The uterus is a thick, hollow, smooth muscle organ consisting of three well-differentiated layers: endometrium, the lining layer consisting of a mucous membrane; myometrium, a thick muscular coat, which is the main segment of the 2 cm thick uterine wall responsible for contractions during labour; and perimetrium, a serosal outer layer (Fig. 5) [62, 63]. The perimetrium protects the uterus and provides a relatively inelastic base upon which the

myometrium develops tension to increase intrauterine pressure. The myometrium consists of four muscle layers separated by a vascular zone. These layers form a web that supports and protects the development of the foetus. Since the uterine muscle layers have different embryonic origins, they have distinct hormonal responses and thus may respond differently to uterotonic agonists and antagonists [63].

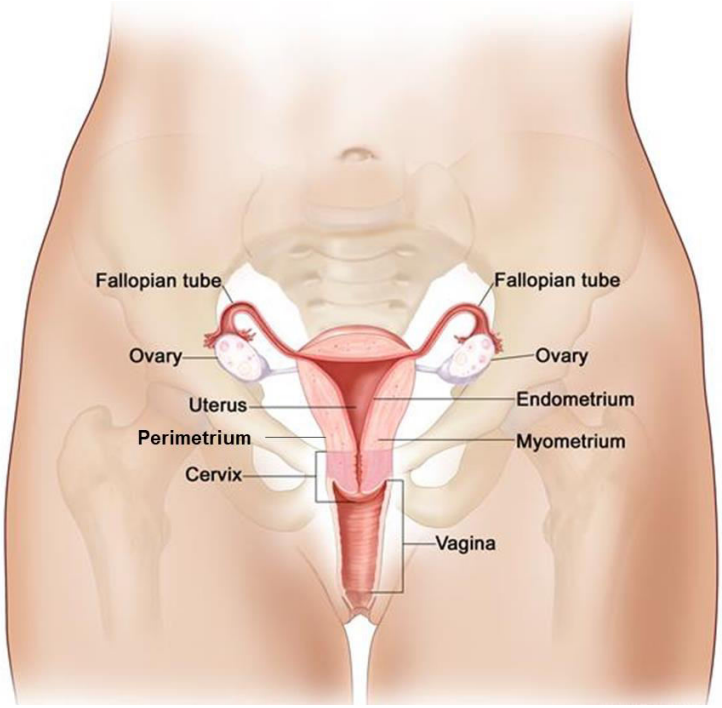


Figure 5. Anatomy of the female reproductive system
The female reproductive system includes the uterus, ovaries, fallopian tubes, cervix, and vagina. The uterus consists of three main layers: perimetrium, the outer layer; myometrium, the layer of smooth muscle cells; and endometrium, which lines the inside of the uterine cavity [64].

1.2.2 Smooth muscle contraction

Uterine contractions occur throughout the menstrual cycle in the non-pregnant state as well as throughout pregnancy. The contractile units of the uterus are the smooth muscle cells in a connective tissue matrix. Movement of contractile forces along the uterus occurs through transmission of tension generated by individual cells to other cells and to the connective tissue [63].

Myometrial cells contain three types of protein myofilaments (actin, myosin, and intermediate filaments), microtubes, and protein structures called dense bodies (Fig. 6A). Actin filaments are composed of congregated, single monomeric actin proteins with six isoforms. The α-actin and γ-actin isoforms are the ones primarily involved in contraction. Actin polymerizes into long, thin (6 to 9 nm) filaments that originate in and are distributed between dense bodies, and slide along the myosin thick filaments to shorten the cell during a contraction (Fig. 6B). Myosin is a hexamer molecule composed of two light chains (MLC) and two heavy chains (MHC), arranged in a head-and-tail structure (Fig. 6C). The MLC has a regulator role in muscle contraction and can bind to calcium and magnesium, and becomes phosphorylated. In addition, the myosin head domain constitutes the 'motor domain' that contains an actin-binding region, as well as the adenosine triphosphate (ATP) hydrolysis site that provides the energy required for force production. Adenosine triphosphatase (ATPase) activity on the myosin head initiates the formation of cross-links or bonds between the actin and myosin filaments, leading to the rotation of myosin heads that pull the actin filaments towards each other, a process leading to the shortening of the cell. This is called the cross bridge cycle and leads to cell contraction [62, 63, 65].

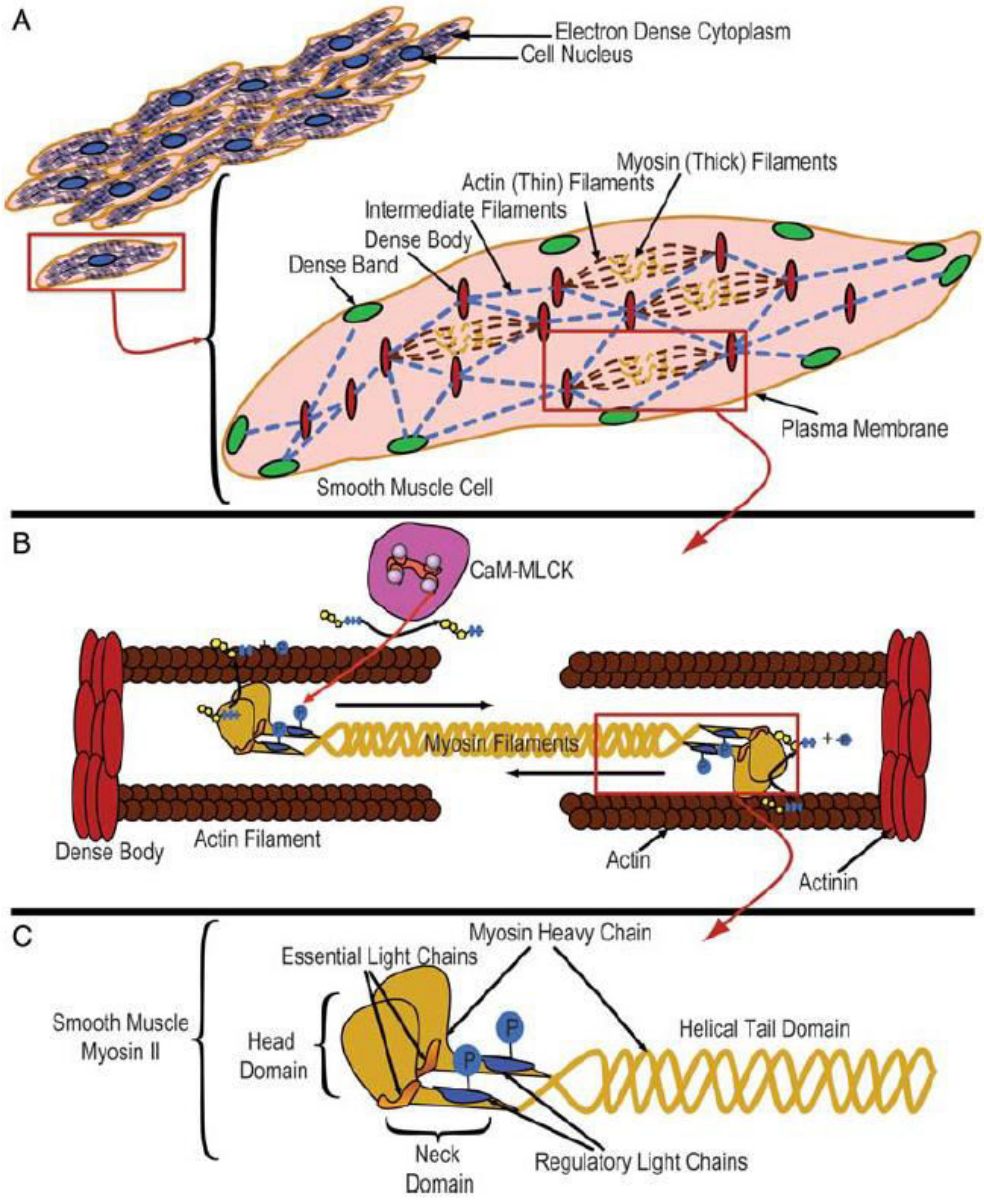


Figure 6. Mechanism of muscle contraction

The intracellular structure of smooth muscle cells is densely packed with components of the contractile machinery. These components include myosin and actin filaments, dense bodies, intermediate filaments and dense band. The network is specifically arranged resulting in force transduction along the longitudinal axis of the cell and cell shortening **(A)**. One unit of the contractile apparatus is composed of two dense bodies with the actin filaments anchored. Myosin is located between the actin filaments **(B)**. Myosin is composed of a head, a neck and a helical tail domain. The regulatory light chains are situated at the neck domain and its phosphorylation causes the formation of a cross bridge between actin and myosin filaments creating a change in the angle of the neck region, and causing motion of the actin filaments that results in shortening of the cell **(C)** [62].

1.3 Preterm labour

During pregnancy, both mother and baby are in a vulnerable state. It is essential for the mother to stay healthy, and potential health issues and illness must be treated to enable a successful pregnancy. On the other hand, the baby needs to be protected from harmful impacts, but also from therapeutic drugs taken by the mother [66]. Unfortunately, problems can arise concerning either the health of the mother or the baby, or in the worst case, becoming life threatening for both. One of the complications that has a great deal of impact, especially on the morbidity and mortality of the new born baby, is PTL [67].

1.3.1 Pathophysiology

Preterm is defined as parturition before 37 weeks of pregnancy. Based on gestational age, preterm birth (PTB) can be divided into sub-categories: extremely preterm, less than 28 weeks; very preterm, between 28 and 32 weeks; and moderate to late preterm, between 32 and 37 weeks [68]. The diagnosis of PTL is often based on clinical criteria of regular contractions accompanied by a change in cervical dilation of at least 2 cm [69]. Preterm occurs for a variety of reasons. Most PTBs occur spontaneously, but some are due to induction of labour or caesarean birth, either for medical or non-medical reasons [68]. Two thirds of PTBs are spontaneous and the other third is induced due to medical indications, such as high blood pressure, preeclampsia, prelabour premature rupture of membranes (PPROM), infection or undersupply of the foetus [70, 71]. However, often no cause is identified [68].

PTB is associated with the majority of all death and chronic disability related to pregnancy, birth and neonatal period [72]. It is a major cause of death and a significant cause of long-term loss of human potential amongst survivors all around the world. Every year, an estimated 15 million babies are born too early, and approximately 1 million die due to complications [68, 73]. Complications of PTB are the largest direct cause of neonatal deaths, and the second most common cause of under-5 year old deaths after pneumonia [73, 74]. Neonatal complications of PTL include respiratory distress syndrome (RDS), sepsis, intraventricular haemorrhage, necrotizing enterocolitis, hypothermia, hypoglycaemia, hyperbilirubinemia, and feeding

problems. Many survivors face a lifetime of disabilities, such as learning disabilities, neurodevelopment impairment, and cerebral palsy [68, 74].

1.3.2 Risks and causes

PTL can be caused by many different processes and is mostly multifactorial [71, 74]. PTB can be classified into two broad subtypes: spontaneous PTB, meaning spontaneous onset of labour or following PPROM; and provider-initiated PTB, defined as induction of labour or elective caesarean birth before 37 weeks of gestation for maternal or foetal indications, or other non-medical reasons [73]. A precise mechanism cannot be established in most cases, therefore several factors that are associated with PTB, but not obvious in the causal pathway, have been sought to explain PTL [70, 72].

Risk factors

Defining risk factors for prediction of PTB allows identification of at-risk women and initiation of risk-specific treatment [70, 72]. Maternal history of PTB is a strong risk factor and most likely driven by the interaction of genetic, epigenetic and environmental risk factors. Some risk factors have been identified, including: maternal age (young or advanced age), short inter-pregnancy interval, medical conditions, psychological factors, and low maternal body mass index (BMI). Another important risk factor is uterine overdistension with multiple pregnancy, which carries nearly 10 times the risk of PTB compared to singleton births [64, 70, 73, 75]. Some lifestyle factors can also contribute to spontaneous PTB, like stress and excessive physical work or long standing times. Smoking and alcohol consumption also have been associated with an increased risk of PTB [73, 76].

Causes

Although PTL presents itself as one clinical manifestation, it cannot be treated by only one therapy or diagnosed by a single method, due to its different aetiologies. For instance, it is not clear whether the mechanism of PTL is premature activation of the common physiological process or a pathophysiological development that leads to an activation of the contractility

apparatus [72, 77]. The precursors to spontaneous PTB vary by gestational age, and social and environmental factors, but the cause of spontaneous PTL remains unidentified in up to half of cases [73, 76]. PTL can be caused by multiple pathologic processes, for example, conditions like short cervix, cervical diseases, preeclampsia, and placental ischaemia [77, 78].

One of the leading causes of PTL is both intra- and extrauterine infection. Intrauterine infection is accountable for at least 25% of PTBs [72, 79]. Several infections, like urinary tract infection, malaria, bacterial vaginosis, human immunodeficiency virus (HIV), and syphilis, are associated with an increased risk of PTB [71, 73, 80, 81]. The colonisation with microorganisms alone is mostly not enough to cause PTL, also an inflammatory response, either from the mother or the foetus, is needed [71]. Inflammation can lead to the activation of the contractility cascade, since pro-inflammatory factors play an important role in the myometrial contractility pathway [62, 82].

1.3.3 Management of preterm labour

Labour is a multifactorial process, and not all aspects of term labour and PTL mechanism are so far known, making the choice of treatment extremely difficult [66, 72, 83]. A delivery delay for at least 48 h may reduce the rate of long-term morbidity by facilitating the maturation of developing organs and systems, and may also permit the transfer of the foetus *in utero* to a centre with a neonatal intensive care unit (NICU). It is important to bear in mind that, between 22 and 28 weeks of gestation, each day of delay increases survival by 3% [74, 84, 85]. Treatments used in clinics address the symptoms rather than the underlying cause. Since the contracting uterus is the most visible symptom of labour, most treatments focus on relaxing the uterus (tocolysis) [66, 69, 71, 86].

1.3.3.1 Foetal lung maturation

In the case of PTB, foetal lung immaturity often leads to RDS, which is mainly accountable for morbidity and mortality in preterm neonates. The administration of antenatal glucocorticoids (AGC) for at least 48 h leads to accelerated lung maturation and reduces the risk of RDS significantly. Treatment with corticosteroids is recommended for all PTL cases between 24 and

34 weeks of gestation [84, 87]. For PTL in pregnancies further advanced than 34 weeks, AGC treatment seems to have no benefit for the mother or for the baby, and is not recommended. Moreover, the use of AGC is not recommended in cases of chorioamnionitis, peptic ulcer (p.o.), and tuberculosis [87]. Besides the improvement of lung maturation, the administration of AGC also reduces the risk of other complications like intraventricular haemorrhage, retinopathy and others [87, 88]. Betamethasone and dexamethasone have been the preferred antenatal treatments [69].

1.3.3.2 Tocolytics

Current approaches to preventing or arresting PTL have been not fully successful. This failure is largely based on poor understanding of the regulation of the timing and maintenance of parturition [89]. Most tocolytics used in clinics were not developed for this purpose and are therefore often not uterospecific, leading to all sorts of side effects [90]. Furthermore, tocolytics are often applied off-label and do not have an official approval for the purpose of tocolysis [85, 91]. There are several tocolytics used in the clinics: β-adrenergic agonists, oxytocin receptor (OTR)-antagonists, calcium-channel blockers (CCBs), prostaglandin (PG)-synthase inhibitors, and progesterone [69, 90].

β-sympathomimetics/-agonists

β-agonists bind to the β-adrenergic receptors located on cardiac/vascular muscle cells, bronchioles of the respiratory system and myometrium (β2-receptors). Relaxation of the smooth muscle occurs when these receptors are activated. β-agonists are efficient in delaying PTL by 48 h, however, due to lack of specificity, they promote several maternal side effects like tachycardia, dyspnoea, palpitation, headache, pulmonary oedema, and hyperglycaemia [66]. Furthermore, since these tocolytics have the ability to pass through the placenta, there is also the possibility that the foetus can experience side effects as well [91].

Oxytocin receptor antagonists

OT is known to play a crucial role in the initiation of labour [65]. A widely used competitive inhibitor of the OTR is atosiban, which binds to the OTR competitively [92]. The arrest of PTL

with atosiban for 48 h is often effective and has fewer side effects than β-adrenergic agonists [93]. Some side effects related to atosiban are nausea, dizziness, headache, and tachycardia [92]. Atosiban is the therapy of choice in most cases, but is also more expensive compared to the other treatment options. However, when considering hospitalisation expenses, total costs are lower than when the other tocolytics are used [94].

Calcium channel blockers

Smooth muscle cell contraction can be caused by the entry of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels (VGCCs) into the intracellular compartment. When these channels are blocked, cells cannot contract [65, 95]. The mostly commonly used CCB to treat PTL in clinics is nifedipine. Nifedipine effectively delays birth for 48 h and is associated with a reduction in the rate of RDS on the premature baby [96]. The use of CCBs leads to fewer side effects than the use of β-agonists [93]. However, given its known use as an antihypertensive drug, it can cause adverse effects like dizziness, flushing, hypotension, and suppression of heart rate, compromising the supply of the foetus [66, 69, 95].

Prostaglandin synthase inhibitors

PG plays a role in the initiation and maintenance of labour [97]. Since PGs are synthesized from arachidonic acid by the cyclooxygenase (COX) enzymes, the inhibition of these enzymes leads to labour inhibition [66]. Maternal side effects include gastritis, oesophageal reflux, nausea, and vomiting. In general, the use of these tocolytics is a safe option for the mother, however, it is only allowed in pregnancies below 32 weeks, due to its risk of causing an early closure of the ductus arteriosus and impairing foetal renal function [90].

Progesterone

The steroid hormone progesterone mediates uterine quiescence before the onset of labour. It is produced by the corpus luteum and, after 8 weeks of gestation, by the placenta [66]. Progesterone acts by binding to the nuclear progesterone receptor, and blocks labour by inhibiting the responsiveness of myometrial cells to pro-labour/pro-inflammatory stimuli [66,

98]. Progesterone is given to the mother, either intramuscularly or intravaginally, as a preventive measure for women at risk, like in cases of previous PTB or short cervix [97].

Other tocolytics

Due to lack of data or negative benefit-risk assessment, several tocolytic treatments are no longer recommended. Magnesium sulphate acts as a Ca^{2+} antagonist, however, its effectiveness is not sufficient to outweigh the potential risks for mother and foetus [69]. In the case of nitric oxide (NO)-donor, there is not sufficient data to recommend its use as a tocolytic, and the maternal vasodilatation can lead to symptoms like flushing, headache, hypotension, and tachycardia [85, 91].

1.4 Contractility pathways

Throughout the journey of pregnancy, the uterus exhibits a relatively quiescent state and responds to a low level of uterotonins until parturition approaches. The beginning of human parturition is clinically manifested by rhythmic uterine contractions, leading to the expulsion of the baby. At a cellular level, the mechanism by which OT leads to stimulation of the uterus is very complex, and consists in the activation of several pathways [62].

1.4.1 Role of oxytocin

The neurohypophysial hormone OT is a peptide consisting of nine amino acids (Fig. 7). This hormone was first discovered by Sir Henry Dale in 1906. The term oxytocin originated from the Greek words '*oxus*' and '*tokos*' meaning 'sharp' and 'childbirth', respectively [99, 100]. OT is mainly produced by the hypothalamus, in the nuclei supraopticus and paraventricularis, and is released by the posterior lobe of the pituitary gland in a pulsatile manner [101], triggering peripheral and central receptors [102]. However, it can also be produced by peripheral tissues like the decidua, placenta or amnion. OT has a major impact on human reproduction, playing a role in the processes of labour, lactation, mother-child bonding and also erectile dysfunction and ejaculation [100, 102].

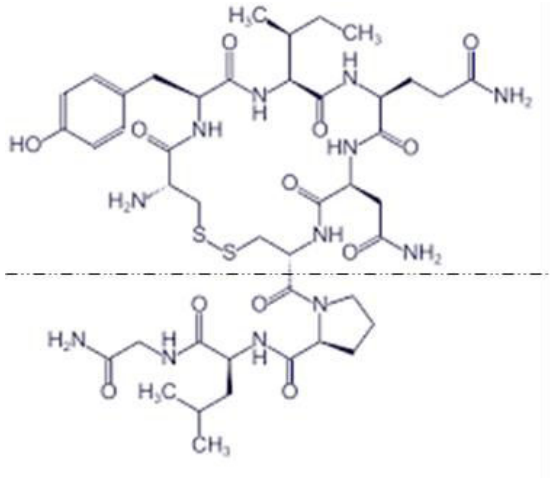


Figure 7. Chemical structure of OT
OT is a nonapeptide hormone composed of a cyclic (above line) and a linear part (below line). Its sequence is cysteine-tyrosine-isoleucine-glutamine-aspargine-cysteine-proline-leucine-glycine-amide (Cys-Tyr-Ile-GLn-Asn-Cys-Pro-Leu-Gly-NH2) [103].

Birth is a complex process based on many biochemical processes. The time of onset of labour is mainly determined by the complex interaction of corticotropin-releasing hormone (CRH) and oestradiol (from the placenta). Instead of upregulating OT production in the hypothalamus, the beginning of parturition is characterised by a higher excitability of the uterine contractility apparatus. In the blood of a pregnant woman, towards the end of pregnancy, the oestradiol level increases by about 150 times, and the progesterone concentration by about 15 times [100]. This oestrogen-progesterone quotient increase is accompanied by an augmentation of the sensitivity of OTRs on myometrium cells. Furthermore, the spontaneous reactivity of the myometrium is increased by an oestrogen-dependent depolarization of the cell membrane and induction of local OT production by the placenta and decidua. Contractions are triggered through the binding of OT to the OTR of myometrial cells [102, 104].

OT is the most potent uterine stimulant known so far, and its pharmacological application is mainly in the induction and augmentation of childbirth or in the prevention of postpartum haemorrhage by stimulating the delivery of the placenta [66, 89].

1.4.2 Oxytocin receptor

OTR functions as a receptor for the hormone OT and belongs to the rhodopsin-type (Class 1) of the guanylate nucleotide binding proteins (G-proteins) coupled receptor (GPCR) family [65, 100, 101] . GPCRs are one of the most important families of cell surface proteins and play a major role in transmembrane signalling [65].

The receptor consists of seven α-helical transmembrane domains connected to the heterodimeric G-proteins. When agonists interact with the OTR, G-protein subunits G_α, G_β and G_γ couple to the receptor to stimulate a number of signalling pathways [65, 89, 104]. A schematic model of the OTR is shown in Figure 8.

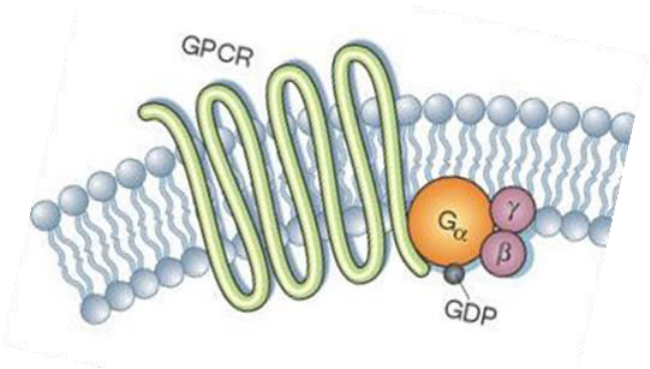


Figure 8. Schematic representation of OTR structure
OTR belongs to the GPCR family, and possesses seven transmembrane domains. The associated G-protein subunits are located in the intracellular compartments. Figure adapted from Li J. *et al.*, 2002 [105].

In the uterus, OTR undergoes a radical up- and down regulation. During pregnancy it is upregulated, to ensure a strong sensitivity towards OT and, after labour, the receptor undergoes a rapid decrease [106]. The tissue specific regulated expression of OTR is essential, since it enables the OT that circulates in the blood to switch target organs and to promote the induction of contractions during labour [100].

1.4.3 Main oxytocin-induced signalling pathways

When OT binds to OTR, a conformational change is induced in the receptor domain. In the myometrium, the OTR has been shown to couple with $G_{\alpha q/11}$, $G_{\alpha i}$ and potentially $G_{\alpha 12/13}$. OTR couples to $G_{\alpha q/11}$ proteins to stimulate membrane phospholipase C-β (PLC-β), promoting its activation. PLC-β then hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 and DAG will act as second messengers, activating different pathways in the cells (Fig. 9) [62, 65, 101].

1.4.3.1 Canonical pathway

IP_3 interacts with a specific receptor (IP_3R) at the level of the sarcoplasmic reticulum (SR), leading to the release of Ca^{2+} from this intracellular store, and increasing the $[Ca^{2+}]_i$ [62]. A further potential way of regulating Ca^{2+} release is referred to as store-operated Ca^{2+} entry (SOCE). As a result of emptying the intracellular SR stores of Ca^{2+}, an unknown signal is sent to the plasma membrane (PM) to allow entry of extracellular Ca^{2+} into the cytosol. The channels through which this entry occurs are referred to as store operated channels (SOCs) [62, 101]. Besides activating the release of intracellular Ca^{2+} storage, OTR stimulation also seems to trigger the opening of VGCCs, also referred to as L-type Ca^{2+} channels [107], implying a further rise in $[Ca^{2+}]_i$. Furthermore, the Ca^{2+}-ATPase pump is inhibited, preventing Ca^{2+} from exiting the cell [100, 101].

Mobilisation of these extra- and intracellular Ca^{2+}-sources trigger the activation of calmodulin (CaM), a Ca^{2+}-dependent cytosolic protein that binds to Ca^{2+} ions. The Ca^{2+}-calmodulin complex (Ca^{2+}-CaM) then activates the myosin light chain kinase (MLCK), leading to the phosphorylation of the MLC initiating the actin-myosin cross-bridge cycle and myometrial contraction (Fig. 9, pink pathway) [62, 100]. Thus, the regulation of the Ca^{2+} flux is of ultimate importance in determining the state of contractile activity. Ca^{2+} is one of the most ubiquitously used second messenger signalling molecules in biological systems [62].

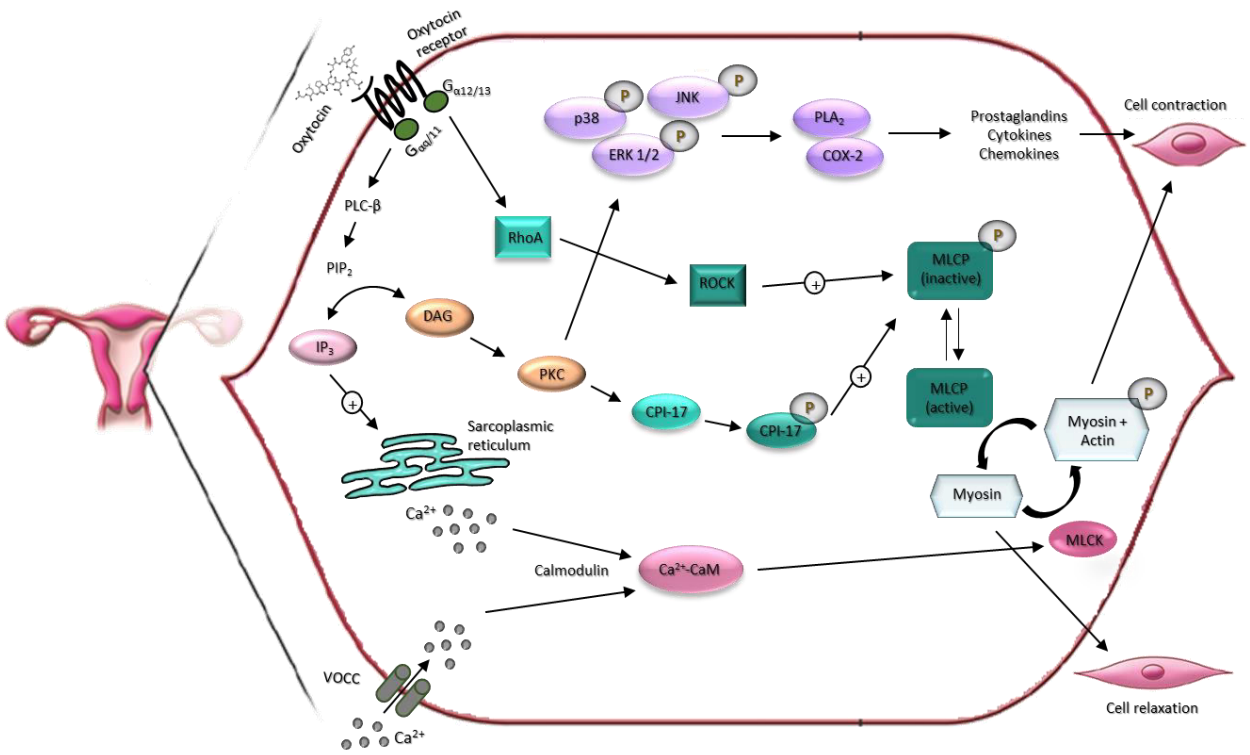


Figure 9. Oxytocin receptor signalling in the myometrium

Binding of OT to its receptor leads to contraction of the myometrium through several pathways. Activation of G-protein $G_{\alpha q/11}$ promotes the activation of phospholipase C-β (PLC-β), leading to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-tirsphosphate (IP_3) and diacylglycerol (DAG). IP_3 then triggers the release of Ca^{2+} from the sarcoplasmic reticulum (SR) which forms a complex with calmodulin, the Ca^{2+}-calmodulin complex (Ca^{2+}-CaM). This complex will activate the myosin light chain kinase (MLCK) leading to the phosphorylation of myosin, enabling it to form the complex myosin-actin cross binding that leads to myometrial contraction. DAG, on the other hand, activates the protein kinase C (PKC), which activates c-kinase-activated protein phosphatase-1 inhibitor 17-kDa (CPI-17) by phosphorylation. CPI-17 then phosphorylates myosin light chain phosphatase (MLCP) that when phosphorylated is inactive, and cannot dephosphorylate myosin, resulting in myometrial contraction. The inactivation of MLCP is also mediated via the RhoA/ROCK pathway. Ras homolog family member A (RhoA) is directly activated by the linkage of OT to the oxytocin receptor (OTR) and leads to the activation of RhoA-associated protein kinase (ROCK), which then also phosphorylates MLCP. Besides CPI-17, PKC also activates mitogen-activated protein kinases (MAPKs), in this case the extracellular-signal regulated kinases (ERK1/2), the p38, and the stress-activated protein kinase or c-Jun NH_2-terminal kinases (SAPK/JNK). These MAPKs lead to upregulation and activation of PG synthesising enzymes such as cyclooxygenase (COX) and cytosolic phospholipase A2 ($cPLA_2$). COX and $cPLA_2$ are responsible for prostaglandin (PG), and inflammatory cytokines and chemokines production, which leads to cell contraction.

1.4.3.2 Calcium sensitization - RhoA/ROCK pathway

In uterine smooth muscle cells, the intensity of myometrial contractions depends on the balance between the activities of MLCK and myosin light chain phosphatase (MLCP), since they dictate the extent of myosin phosphorylation and therefore myometrial contraction [101]. Inactivation of MLCP by phosphorylation allows MLC to remain phosphorylated and leads to uterine muscle contraction. If MLCP is in its active, dephosphorylated form, it leads to the dephosphorylation of the MLC and therefore also to the disruption of the cross-bridge cycle and myometrium relaxation [62, 101].

MLCP is composed of three subunits: the catalytic region (protein phosphatase 1 or PP1), the myosin phosphatase target subunit 1 (MYPT1) and a third small peptide of approximately 20 kDa (M20) of unknown function [62, 89]. The most relevant subunit for the function of MLCP is MYPT1, whose main objective is to provide access to the target of dephosphorylation of the phosphorylated MLC. MYPT1 has two major phosphorylation sites (Thr^{696} and Thr^{853}) and by the phosphorylation of MYPT1, MLCP's ability to dephosphorylate MLC is inhibited [62].

DAG, one of the second messengers resulting from PIP_2 hydrolysis, activates the protein kinase C (PKC) isoforms [62], leading to the phosphorylation of c-kinase-activated protein phosphatase-1 inhibitor 17-kDa (CPI-17), a smooth muscle specific inhibitor of MLCP. Phosphorylated CPI-17 promotes MLCP phosphorylation and, therefore, impedes its relaxation effect on the myometrium [101, 108].

Ras homolog family member A (RhoA) is a monomeric G protein and a member of the Rho subfamily of the Ras G superfamily of monomeric guanosine triphosphatases (GTPases). RhoA exists in an inactive form in the cytosol bound to guanosine diphosphate (GDP) in a complex including GDP-dissociation inhibitor (GDI). To be activated, this complex is disrupted and the GDP exchanged for guanosine triphosphate (GTP) [89, 101]. In parallel to the PKC pathway, MLCP is also affected via the RhoA/ROCK pathway [62, 109]. The binding of OT to its receptor also leads to the activation of $G_{\alpha 12/13}$ that triggers the activation of RhoA [101]. The binding of GTP-RhoA to the binding domain of RhoA-associated kinase (ROCK) leads to a conformational change causing autophosphorylation and activation of ROCK. Like PKC, ROCK can inactivate MLCP by phosphorylation, thereby shifting the balance of MLCP and MLCK activity to the side

that benefits myometrial contractility [89, 101, 109, 110]. This promotion of contractility is called calcium sensitization, a term that implies an indirect effect on the Ca^{2+}-dependent pathway, increasing the effect of the available Ca^{2+} on the contractility apparatus by displacing the equilibrium to the MLCK side (Fig. 9, orange and green pathway) [65].

1.4.3.3 MAPK pathway

In addition to the well characterised, direct or indirect, Ca^{2+}-dependent pathways, there is also a signalling segment independent of Ca^{2+}. Previous studies suggest that OT also acts as an inflammatory mediator, playing a central role in the inflammatory cascade leading to labour, by activating mitogen-activated protein kinases (MAPK) [99, 111]. The MAPK pathway is a highly conserved signal transduction pathway in all eukaryotic cells, and one of the best-characterized signalling cascades [112].

In multicellular organisms there are three major classes of MAPKs: the extracellular-signal regulated kinases (ERK1 and ERK2); the four p38 enzymes, p38α, p38β, p38γ, and p38δ; and the stress-activated protein kinase or c-Jun NH_2-terminal kinases (SAPK/JNK), JNK 1, JNK2, and JNK3. The three MAPK classes play an important role in cellular regulation processes, especially on gene expression, mitosis, movement, metabolism and programmed cell death [113-115]. Spatial location of the MAPK ERK1/2 determines target substrates and later effects within the cell: i) nuclear translocation of activated ERK is followed by upregulation of gene expression, and ii) activated ERK remaining in the cytoplasm leads to contraction and PG production [112, 116]. MAPKs SAPK/JNK, when activated, can translocate into the nucleus and bind to the deoxyribonucleic acid (DNA) binding protein c-Jun, increasing its transcriptional activity. This has marked consequences, as c-Jun is a component of the activator protein 1 (AP-1), an important regulator of gene expression that contributes to the control of many cytokine genes [99, 114]. Both SAPK/JNK and p38 MAPKs play an important role in apoptosis, inflammation, and cytokine production [82, 113, 115].

The MAPK cascade is regulated as a three-kinase system including MAPK, activated by MAPK kinase (MKK), which is in turn activated by MKK kinase (MKKK). For a MAPK to be activated, two sites have to be phosphorylated (serine and threonine). The activation of MAPKs is reversed by MAPK phosphatases [113-115]. In the uterus, activated MAPKs lead to upregulation and

activation of PG synthesising enzymes, such as cytosolic phospholipase A_2 ($cPLA_2$) and COX isoforms (COX 1 and 2). All three classes of MAPK have been reported to phosphorylate $cPLA_2$ [117, 118]. MAPKs phosphorylate $cPLA_2$ at $Serine^{505}$, leading to selective hydrolysis of glycerol-phospholipids, and mobilising arachidonic acid from membrane phospholipids [119, 120]. Once mobilised, free arachidonic acid is rapidly converted to prostaglandin H_2 (PGH_2) by the enzyme PGH_2 endoperoxide synthase (PGHS)-2. In endometrial tissue, PGH_2 is then converted to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and E (PGE_2) by the enzyme PGF synthase [121]. COX, also known as PGHS or prostaglandin-endoperoxide synthase (PTGS), is also responsible for PG production (Fig. 9, orange and purple pathway) [99, 111, 122]. PGs have a pro-inflammatory effect on the myometrial tissue and lead to contractions by increasing Ca^{2+} entry [66]. This pro-inflammatory pathway that leads to PG production is also present in other uterine tissues like the amnion and the decidua [101, 123].

1.5 References

1. Nagaratna A, and Hegde PL (2015). A comprehensive review on Parnabeeja [*Bryophyllum pinnatum* (Lam.) Oken]. *J Med Plants Stud*. 3(5): 166-71.
2. Kamboj A, and Saluja AK (2009). *Bryophyllum pinnatum* (Lam.) Kurz.: phytochemical and pharmacological profile: a review. *Pharmacogn Rev*. 3(6): 364-374.
3. *Bryophyllum pinnatum* plant. Accessed on 14 April 2020. Available from: https://livegarden.in/product/siam-garden-panfuti-bryophyllum-pinnatum-miracle-leaf-plant-patharchatta-kidney-stone-medicinal-ayurvedic-plant-.
4. Conrad's J. *Bryophyllum pinnatum*. Accessed on 14 April 2020. Available from: https://www.backyardnature.net/mexnat/airplant.htm.
5. *Bryophyllum pinnatum*. Accessed on 14 April 2020. Available from: https://commons.wikimedia.org/wiki/File:Life_plant_(Bryophyllum_pinnatum)_flowers.jpg.
6. Salisbury RA, and Hooker W. *The Paradisus Londinensis: or Coloured Figures of Plants Cultived in the Vicinity of the Metropolis*. 1805, William Hoocker. p. 155.
7. Hamet R, *Monographie du genre Kalanchoe*, in *Bulletin de l'Herbier Boissier*. 1907. p. 869-900.
8. Boiteau P, and Mannoni O. *Les Kalanchoe (suite)*. 1948: Cactus. p. 7-10.
9. Berger A, and Harms AT. *Crassulaceae*. 1930, Adolf Engler: The natural plants families. p. 352-485.
10. Fürer K, Raith M, Brenneisen R, Mennet M, Simões-Wüst AP, von Mandach U, Hamburger M, and Potterat O (2013). Two new flavonol glycosides and a metabolite profile of *Bryophyllum pinnatum*, a phytotherapeutic used in obstetrics and gynaecology. *Planta Medica*. 79(16): 1565-1571.
11. Kamboj A, Rathour A, and Kaur M (2013). Bufadienolides and their medicinal utility: A review. *Int J Pharm Pharm Sci*. 5: 20-27.
12. Kolodziejczyk-Czepas J, and Stochmal A (2017). Bufadienolides of *Kalanchoe* species: an overview of chemical structure, biological activity and prospects for pharmacological use. *Phytochem Rev*. 16(6): 1155-1171.
13. Gao H, Popescu R, Kopp B, and Wang Z (2011). Bufadienolides and their antitumor activity. *Nat Prod Rep*. 28(5): 953-969.
14. Wagner H, Lotter H, and Fischer M (1986). Die toxischen und sedierend wirkenden Bufadienolide von *Kalanchoe daigremontiana* Hamet et Perr. *Helv Chim Acta*. 69(2): 359-367.
15. Supratman U, Fujita T, Akiyama K, and Hayashi H (2000). New insecticidal bufadienolide, bryophyllin C, from *Kalanchoe pinnata*. *Biosci Biotechnol Biochem*. 64(6): 1310-1312.
16. McKenzie R, Franke F, and Dunster P (1987). The toxicity to cattle and bufadienolide content of six Bryophyllum species. *Aust Vet J*. 64(10): 298-301.

17. Fürer K, Simões-Wüst AP, von Mandach U, Hamburger M, and Potterat O (2016). *Bryophyllum pinnatum* and related species used in anthroposophic medicine: constituents, pharmacological activities, and clinical efficacy. *Planta Medica*. 82(11/12): 930-941.

18. Havsteen B (1983). Flavonoids, a class of natural products of high pharmacological potency. *Biochem Pharmacol*. 32(7): 1141-1148.

19. Elliott M, and Chithan K. *The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer*, in *The flavonoids advances in research since 1986*. 2017, Routledge. p. 619-652.

20. Grange J, and Davey R (1990). Antibacterial properties of propolis (bee glue). *J Roy Soc Med*. 83(3): 159-160.

21. Cushnie TT, and Lamb AJ (2005). Antimicrobial activity of flavonoids. *Int J Antimicrob Agents*. 26(5): 343-356.

22. Harborne JB, and Williams CA (2000). Advances in flavonoid research since 1992. *Phytochemistry*. 55(6): 481-504.

23. Havsteen BH (2002). The biochemistry and medical significance of the flavonoids. *Pharmacol Therapeut*. 96(2): 67-202.

24. Fürer K, Eberli D, Betschart C, Brenneisen R, De Mieri M, Hamburger M, Mennet-von Eiff M, Potterat O, Schnelle M, Simões-Wüst A, and von Mandach U (2015). Inhibition of porcine detrusor contractility by the flavonoid fraction of *Bryophyllum pinnatum* – a potential phytotherapeutic drug for the treatment of the overactive bladder syndrome. *Phytomedicine*. 22(1): 158-164.

25. Bachmann S, Betschart C, Gerber J, Fürer K, Mennet M, Hamburger M, Potterat O, von Mandach U, and Simões-Wüst AP (2017). Potential of *Bryophyllum pinnatum* as a detrusor relaxant: an in vitro exploratory study. *Planta Medica*. 83(16): 1274-1280.

26. Lindenberg C. *Rudolf Steiner - a biography*. 2012.

27. Steiner R. *The story of my life*. 1928.

28. Kienle GS, Albonico H-U, Baars E, Hamre HJ, Zimmermann P, and Kiene H (2013). Anthroposophic medicine: an integrative medical system originating in Europe. *Glob Adv Health Med*. 2(6): 20-31.

29. Hamburger M, Potterat O, Furer K, Simoes-Wüst AP, and von Mandach U (2017). *Bryophyllum pinnatum*-Reverse Engineering of an Anthroposophic Herbal Medicine. *Nat Prod Commun*. 12(8): 1359-1364.

30. von Mandach U, and Betschart C (2011). *Bryophyllum pinnatum* in der Geburtshilfe und Gynäkologie. Wirksamkeit und Verträglichkeit. *info@ gynäkologie*(3): p. 16-20.

31. Hassauer W, Schreiber K, and Von der Decken D (1985). Ein neuer Weg in der tokolytischen Therapie. *Erfahrungsheikunde*. 34: 683-687.

32. Simões-Wüst AP, Jeschke E, Mennet M, Schnelle M, Matthes H, and von Mandach U (2012). Prescribing pattern of *Bryophyllum* preparations among a network of anthroposophic physicians. *Complement Med Res*. 19(6): 293-301.

33. Fürer K, Simões-Wüst AP, Winkler A, Amsler N, Schnelle M, and von Mandach U (2015). Die Anwendung von *Bryophyllum pinnatum* - Präparaten in der Geburtshilfe und Gynäkologie-eine multizentrische prospektive Beobachtungsstudie. *Complement Med Res*. 22(4): 231-236.

34. Schenkel L, Simões-Wüst AP, Hoesli I, and von Mandach U (2018). Drugs in pregnancy and lactation - medications used in Swiss obstetrics. *Z Geburtsh Neonatol*. 222: 1-14.

35. Daub E, *Vorzeitige Wehentätigkeit: ihre Behandlung mit pflanzlichen Substanzen: eine klinische Studie*. 1989: Urachhaus.

36. Vilàghy (2002). Decrease in the incidence of premature deliveries with the use of phytotherapy–Results of clinical practice. *Therapeutische Umschau*. 59(12): 696-701.

37. Gwehenberger B, Rist L, Huch R, and von Mandach U (2004). Effect of *Bryophyllum pinnatum* versus fenoterol on uterine contractility. *Eur J Obstet Gynecol Reprod Biol*. 113(2): 164-171.

38. Plangger N, Rist L, Zimmermann R, and von Mandach U (2006). Intravenous tocolysis with *Bryophyllum pinnatum* is better tolerated than beta-agonist application. *Eur J Obstet Gynecol Reprod Biol*. 124(2): 168-172.

39. Simões-Wüst AP, Grãos M, Duarte C, Brenneisen R, Hamburger M, Mennet M, Ramos M, Schnelle M, Wächter R, and Worel A (2010). Juice of *Bryophyllum pinnatum* (Lam.) inhibits oxytocin-induced increase of the intracellular calcium concentration in human myometrial cells. *Phytomedicine*. 17(12): 980-986.

40. Wächter R, Brenneisen R, Hamburger M, Mennet M, Schnelle M, Worel A, Simões-Wüst AP, and Von Mandach U (2011). Leaf press juice from *Bryophyllum pinnatum* (Lamarck) Oken induces myometrial relaxation. *Phytomedicine*. 19(1): 74-82.

41. Haylen BT, De Ridder D, Freeman RM, Swift SE, Berghmans B, Lee J, Monga A, Petri E, Rizk DE, and Sand PK (2010). An International Urogynecological Association (IUGA)/International Continence Society (ICS) joint report on the terminology for female pelvic floor dysfunction. *Neurourol Urodyn*. 29(1): 4-20.

42. Schuler V, Suter K, Fürer K, Eberli D, Horst M, Betschart C, Brenneisen R, Hamburger M, Mennet M, and Schnelle M (2012). *Bryophyllum pinnatum* inhibits detrusor contractility in porcine bladder strips—A pharmacological study towards a new treatment option of overactive bladder. *Phytomedicine*. 19(10): 947-951.

43. Betschart C, von Mandach U, Seifert B, Scheiner D, Perucchini D, Fink D, and Geissbühler V (2013). Randomized, double-blind placebo-controlled trial with *Bryophyllum pinnatum* versus placebo for the treatment of overactive bladder in postmenopausal women. *Phytomedicine*. 20(3): 351-358.

44. Lambrigger-Steiner C, Simões-Wüst A, Kuck A, Fürer K, Hamburger M, and Von Mandach U (2014). Sleep quality in pregnancy during treatment with *Bryophyllum pinnatum*: an observational study. *Phytomedicine*. 21(5): 753-757.

45. Simões-Wüst AP, Al Hassani T, Müller-Hübenthal B, Pittl S, Kuck A, Meden H, Eberhard J, Decker M, Fürer K, and von Mandach U (2015). Sleep quality improves during treatment with *Bryophyllum pinnatum* an observational study on cancer patients. *Integr Cancer Ther*. 14(5): 452-459.

46. Biswas SK, Chowdhury A, Das J, Hosen SZ, Uddin R, and Rahaman MS (2011). Literature review on pharmacological potentials of *Kalanchoe pinnata* (Crassulaceae). *Afr J Pharm Pharmacol*. 5(10): 1258-1262.

47. Pal S, Sen T, and Chaudhuri A (1999). Neuropsychopharmacological profile of the methanolic fraction of Bryophyllum pinnatum leaf extract. *J Pharm Pharmacol*. 51(3): 313-318.

48. Yemitan O, and Salahdeen H (2005). Neurosedative and muscle relaxant activities of aqueous extract of Bryophyllum pinnatum. *Fitoterapia*. 76(2): 187-193.

49. Salahdeen H, and Yemitan O (2006). Neuropharmacological effects of aqueous leaf extract of Bryophyllum pinnatum in mice. *Afr J Biomed Res*. 9(2).

50. Yamagishi T, Haruna M, Yan X-Z, Chang J-J, and Lee K-H (1989). Antitumor agents, 110, Bryophyllin B, a novel potent cytotoxic bufadienolide from Bryophyllum pinnatum. *J Nat Prod*. 52(5): 1071-1079.

51. Supratman U, Fujita T, Akiyama K, Hayashi H, Murakami A, Sakai H, Koshimizu K, and Ohigashi H (2001). Anti-tumor Promoting Activity of Bufadienolides from Kalanchoe pinnata and K. daigremontiana× butiflora. *Biosci Biotechnol Biochem*. 65(4): 947-949.

52. Mahata S, Maru S, Shukla S, Pandey A, Mugesh G, Das BC, and Bharti AC (2012). Anticancer property of Bryophyllum pinnata (Lam.) Oken. leaf on human cervical cancer cells. *BMC Complement Altern Med*. 12(1): 15.

53. Ojewole JA (2005). Antinociceptive, anti-inflammatory and antidiabetic effects of *Bryophyllum pinnatum* (Crassulaceae) leaf aqueous extract. *J Ethnopharmacol*. 99(1): 13-19.

54. Afzal M, Gupta G, Kazmi I, Rahman M, Afzal O, Alam J, Hakeem KR, Pravez M, Gupta R, and Anwar FJF (2012). Anti-inflammatory and analgesic potential of a novel steroidal derivative from *Bryophyllum pinnatum*. *Phytomedicine* 83(5): 853-858.

55. Chibli LA, Rodrigues KC, Gasparetto CM, Pinto NC, Fabri RL, Scio E, Alves MS, Del-Vechio-Vieira G, and Sousa OV (2014). Anti-inflammatory effects of Bryophyllum pinnatum (Lam.) Oken ethanol extract in acute and chronic cutaneous inflammation. *J Ethnopharmacol*. 154(2): 330-338.

56. Omojokun OS, Oboh G, Ademiluyi AO, Oladele JO, and Boligon AA (2020). Impact of drying processes on Bryophyllum pinnatum phenolic constituents and its anti-inflammatory and antioxidative activities in human erythrocytes. *J Food Biochem*: p. e13298.

57. Wächter R, *Klinische Wirksamkeit, Pharmakologie und Analytik von Bryophyllum pinnatum*, in *Division of Pharmaceutical Biology*. 2010, University of Basel, Basel.

58. Hosomi JK, Ghelman R, Quintino MP, de Souza E, Nakamura MU, and Moron AF (2014). Effects of chronic *Bryophyllum pinnatum* administration on Wistar rat pregnancy. *Forsch Komplementmed*. 21(3): 184-189.

59. Devbhuti D, Gupta JK, Devbhuti P, and Bose A (2008). Phytochemical and acute toxicity study on *Bryophyllum calycinum* SALISB. *Acta Pol Pharm*. 65(4): 501-504.

60. Kitzinger S, *The complete book of pregnancy & childbirth*. 2004: Knopf.

61. Chakravarti S, Pai M, and Kushtagi P, *Manual of Obstetrics E-book*. 2015: Elsevier Health Sciences.

62. Aguilar HN, and Mitchell B (2010). Physiological pathways and molecular mechanisms regulating uterine contractility. *Hum Reprod Update*. 16(6): 725-744.

63. Blackburn S, *Maternal, Fetal, & neonatal physiology-E-book: a clinical perspective*. 2017: Elsevier Health Sciences.

64. National Cancer Institute. Available from: https://www.cancer.gov/publications/dictionaries/cancer-terms/def/uterus.

65. Arrowsmith S (2020). Oxytocin and vasopressin signalling and myometrial contraction. *Curr Opin Physiol*. 13: 62-70.

66. Arrowsmith S, Kendrick A, and Wray S (2010). Drugs acting on the pregnant uterus. *Obstet Gynaecol Reprod Med*. 20(8): 241-247.

67. National Institute for Health and Care Excellence (NICE), *Preterm labour and birth*. 2015: NICE: National Institute for Health and Care Excellence.

68. WHO: World Health Organization. Accessed on April 11. Available from: http://www.who.int/mediacentre/factsheets/fs363/en/.

69. ACOG: American College of Obstetricians and Ginecologists (2016). Practice Bulletin No. 171: Management of Preterm Labor. *Obstet Gynecol*. 128(4): p. e155.

70. Goldenberg RL, Culhane JF, Iams JD, and Romero R (2008). Epidemiology and causes of preterm birth. *The lancet*. 371(9606): 75-84.

71. Romero R, Dey SK, and Fisher SJ (2014). Preterm labor: one syndrome, many causes. *Science*. 345(6198): 760-765.

72. Vogel JP, Chawanpaiboon S, Moller A-B, Watananirun K, Bonet M, and Lumbiganon P (2018). The global epidemiology of preterm birth. *Best Pract Res Clin Obstet Gynaecol*. 52: 3-12.

73. Blencowe H, Cousens S, Chou D, Oestergaard M, Say L, Moller A-B, Kinney M, and Lawn J (2013). Born too soon: the global epidemiology of 15 million preterm births. *Reprod Health*. 10(1): S2.

74. Manuck TA, Rice MM, Bailit JL, Grobman WA, Reddy UM, Wapner RJ, Thorp JM, Caritis SN, Prasad M, and Tita AT (2016). Preterm neonatal morbidity and mortality by gestational age: a contemporary cohort. *Am J Obstet Gynecol*. 215(1): p. 103. e1-103. e14.

75. Heino A, Gissler M, Hindori-Mohangoo AD, Blondel B, Klungsøyr K, Verdenik I, Mierzejewska E, Velebil P, Sól Ólafsdóttir H, and Macfarlane A (2016). Variations in multiple birth rates and impact on perinatal outcomes in Europe. *PloS one*. 11(3): p. e0149252.

76. Waldenström U, Aasheim V, Nilsen ABV, Rasmussen S, Pettersson HJ, and Shytt E (2014). Adverse pregnancy outcomes related to advanced maternal age compared with smoking and being overweight. *Obstet Gynecol*. 123(1): 104-112.

77. Romero R, Espinoza J, Kusanovic JP, Gotsch F, Hassan S, Erez O, Chaiworapongsa T, and Mazor M (2006). The preterm parturition syndrome. *BJOG: Int J Obstet Gynaecol*. 113: 17-42.

78. Barros-Silva J, Pedrosa AC, and Matias A (2014). Sonographic measurement of cervical length as a predictor of preterm delivery: a systematic review. *J Perinat Med*. 42(3): 281-293.

79. Gonçalves LF, Chaiworapongsa T, and Romero R (2002). Intrauterine infection and prematurity. *Ment Retard Dev Disabil Res Rev*. 8(1): 3-13.

80. Haahr T, Ersbøll AS, Karlsen MA, Svare J, Sneider K, Hee L, Weile LK, Ziobrowska-Bech A, Østergaard C, and Jensen JS (2016). Treatment of bacterial vaginosis in pregnancy in order to reduce the risk of spontaneous preterm delivery–a clinical recommendation. *Acta Obstet Gynecol Scand*. 95(8): 850-860.

81. Amabebe E, Reynolds S, He X, Wood R, Stern V, and Anumba DO (2019). Infection/inflammation-associated preterm delivery within 14 days of presentation with symptoms of preterm labour: A multivariate predictive model. *PLoS One*. 14(9): p. e0222455.

82. Kim SH, MacIntyre DA, Da Silva MF, Blanks AM, Lee YS, Thornton S, Bennett PR, and Terzidou V (2015). Oxytocin activates NF-κB-mediated inflammatory pathways in human gestational tissues. *Mol Cell Endocrinol*. 403: 64-77.

83. Muglia LJ, and Katz M (2010). The enigma of spontaneous preterm birth. *New England J Med*. 362(6): 529-535.

84. Di Renzo GC, Al Saleh E, Mattei A, Koutras I, Clerici G (2006). Use of tocolytics: what is the benefit of gaining 48 hours for the fetus? *BJOG: Int J Obstet Gynaecol*. 113: 72-77.

85. Hösli I, Sperschneider C, Drack G, Zimmermann R, Surbek D, and Irion O (2014). Tocolysis for preterm labor: Expert opinion. *Arch Gynecol Obstet*. 289(4): 903-909.

86. Schleußner E (2013). The prevention, diagnosis and treatment of premature labor. *Dtsch Ärztebl Int*. 110(13): p. 227.

87. Roberts D, Brown J, Medley N, and Dalziel SR (2017). Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database Syst Rev*(3).

88. Halliday H (2000). Costs and benefits of perinatal corticosteroid treatment. *Prenat Neonat Med*. 5(4): 201-203.

89. Arthur P, Taggart M, and Mitchell B (2007). Oxytocin and parturition: a role for increased myometrial calcium and calcium sensitization? *Front Biosci*. 12: p. 619.

90. Lamont CD, Jorgensen JS, and Lamont R (2016). The safety of tocolytics used for the inhibition of preterm labour. *Expert Opin Drug Saf*. 15(9): 1163-1173.

91. Haram K, Mortensen JHS, and Morrison JC (2015). Tocolysis for acute preterm labor: does anything work. *J Matern Fetal Neonatal Med*. 28(4): 371-378.

92. Flenady V, Reinebrant HE, Liley HG, Tambimuttu EG, and Papatsonis DN (2014 Jun 6). Oxytocin receptor antagonists for inhibiting preterm labour. *Cochrane Database Syst Rev* (6): p. CD004452.

93. Haas DM, Imperiale TF, Kirkpatrick PR, Klein RW, Zollinger TW, and Golichowski AM (2009). Tocolytic therapy: a meta-analysis and decision analysis. *Obstet Gynecol*. 113(3): 585-594.

94. Wex J, Connolly M, and Rath W (2009). Atosiban versus betamimetics in the treatment of preterm labour in Germany: an economic evaluation. *BMC Pregnancy Childbirth*. 9(1): p. 23.

95. Gáspár R, and Hajagos-Tóth J (2013). Calcium channel blockers as tocolytics: principles of their actions, adverse effects and therapeutic combinations. *Pharmaceuticals*. 6(6): 689-699.

96. Flenady V, Wojcieszek AM, Papatsonis DNM, Stock OM, Murray L, Jardine LA, and Carbonne B (2014 Jun 5). Calcium channel blockers for inhibiting preterm labour and birth. *Cochrane Database Syst Rev* (6): p. CD002255.

97. Hubinont C, and Debiève F (2011). Prevention of preterm labour: 2011 update on tocolysis. *J Pregnancy*.

98. Amini P, Wilson R, Wang J, Tan H, Yi L, Koeblitz WK, Stanfield Z, Romani AM, Malemud CJ, Mesiano SJM, and endocrinology c (2019). Progesterone and cAMP synergize to inhibit responsiveness of myometrial cells to pro-inflammatory/pro-labor stimuli. *Mol Cell Endocrinol.* 479: 1-11.

99. Kim SH, Bennett PR, and Terzidou V (2017). Advances in the role of oxytocin receptors in human parturition. *Mol Cell Endocrinol*. 449: 56-63.

100. Gimpl G, and Fahrenholz F (2001). The oxytocin receptor system: structure, function, and regulation. *Physiol Rev*. 81(2): 629-683.

101. Arrowsmith S, and Wray S (2014). Oxytocin: its mechanism of action and receptor signalling in the myometrium. *J Neuroendocrinol*. 26(6): 356-369.

102. Bethlehem RA, van Honk J, Auyeung B, and Baron-Cohen S (2013). Oxytocin, brain physiology, and functional connectivity: a review of intranasal oxytocin fMRI studies. *Psychoneuroendocrinology*. 38(7): 962-974.

103. Oikonen V. Oxytocin and oxytocin receptors. Accessed on 11 July 2020. Available from: http://www.turkupetcentre.net/petanalysis/target_oxytocin.html.

104. Fuchs A-R (2013). The role of oxytocin in parturition. *Curr Top Exp Endocrinol*. 4: 231-265.

105. Li J, Ning Y, Hedley W, Saunders B, Chen Y, Tindill N, Hannay T, and Subramaniam S (2002). The molecule pages database. *Nature*. 420(6916): 716-717.

106. Blanks AM, and Thornton S (2003). The role of oxytocin in parturition. *BJOG: Int J Obstet Gynaecol*. 110(s20): 46-51.

107. Wray S, Kupittayanant S, Shmygol A, Smith R, and Burdyga T (2001). The physiological basis of uterine contractility: a short review. *Exp Physiol*. 86(02): 239-246.

108. Ozaki H, Yasuda K, Kim YS, Egawa M, Kanzaki H, Nakazawa H, Hori M, Seto M, and Karaki H (2003). Possible role of the protein kinase C/CPI-17 pathway in the augmented contraction of human myometrium after gestation. *Br J Pharmacol*. 140(7): 1303-1312.

109. Tahara M, Morishige K-i, Sawada K, Ikebuchi Y, Kawagishi R, Tasaka K, and Murata Y (2002). RhoA/Rho-kinase cascade is involved in oxytocin-induced rat uterine contraction. *Endocrinology*. 143(3): 920-929.

110. Lartey J, and Bernal AL (2009). RHO protein regulation of contraction in the human uterus. *Reproduction*. 138(3): 407-424.

111. Molnár M, Rigó Jr J, Romero R, Hertelendy FJAjoo, and gynecology (1999). Oxytocin activates mitogen-activated protein kinase and up-regulates cyclooxygenase-2 and prostaglandin production in human myometrial cells. *AJOG: Am J Obstet Gynecol.* 181(1): 42-49.

112. Burotto M, Chiou VL, Lee JM, and Kohn EC (2014). The MAPK pathway across different malignancies: a new perspective. *Cancer*. 120(22): 3446-3456.

113. Cuenda A, and Rousseau S (2007). p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim Biophys Acta*. 1773(8): 1358-1375.

114. Johnson GL, and Lapadat RJS (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science.* 298(5600): 1911-1912.

115. Morrison DK (2012). MAP kinase pathways. *Cold Spring Harb Perspect Biol*. 4(11): p. a011254.

116. Devost D, Wrzal P, and Zingg HH (2008). Oxytocin receptor signalling. *Prog Brain Res*. 170: 167-176.

117. Hazan-Halevy I, and Levy R, *Activation of cytosolic phospholipase A 2 by opsonized zymosan in human neutrophils requires both ERK and p38 MAP-kinase*, in *The Biology and Pathology of Innate Immunity Mechanisms*. 2002, Springer. p. 115-123.

118. Putten VV, Refaat Z, Dessev C, Blaine S, Wick M, Butterfield L, Han S-Y, Heasley LE, and Nemenoff RA (2001). Mechanisms of Signal Transduction-Induction of cytosolic phospholipase A2 by oncogenic Ras is mediated through the JNK and ERR pathways in rat epithelial cells. *J Biol Chem*. 276(2): 1226-1232.

119. Kramer RM, and Sharp JD (1997). Structure, function and regulation of Ca2+-sensitive cytosolic phospholipase A2 (cPLA2). *FEBS letters*. 410(1): p. 49-53.

120. Leslie CC (1997). Properties and regulation of cytosolic phospholipase A2. *J Biol Chem*. 272(27): p. 16709-16712.

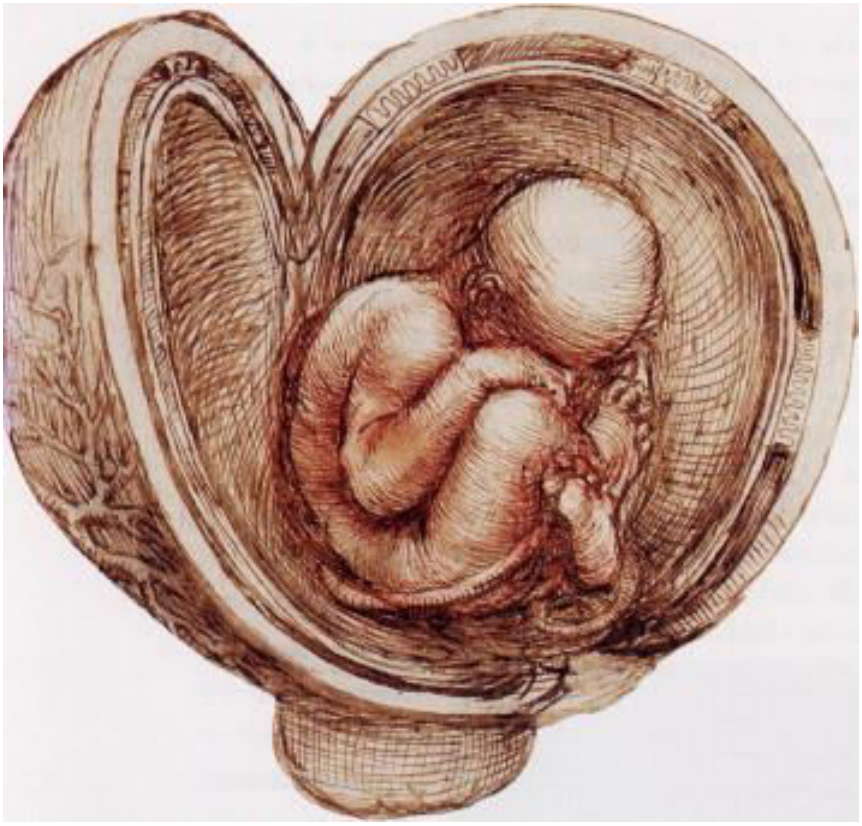
121. Smith WL (1989). The eicosanoids and their biochemical mechanisms of action. *Biochem J*. 259(2): 315.

122. Khan AH, Carson RJ, and Nelson SMJFB (2008). Prostaglandins in labor--a translational approach. *Front Biosci.* 13: p. 5794-5809.

123. Kim SH, Pohl O, Chollet A, Gotteland J-P, Fairhurst AD, Bennett PR, and Terzidou V (2017). Differential effects of oxytocin receptor antagonists, Atosiban and Nolasiban, on OT-mediated signalling in human amnion and myometrium. *Mol Pharmacol*. 91(4): 403-415.

Chapter 2

Myometrium contractility



Sketch by Leonardo da Vinci of uterus with foetus, circa 1510.

2.1. *Bryophyllum pinnatum* enhances the inhibitory effect of atosiban and nifedipine on human myometrial contractility: an *in vitro* study

Stefanie Santos, Christian Haslinger, Mónica Mennet, Ursula von Mandach, Matthias Hamburger, Ana Paula Simões-Wüst

BMC Complementary and Alternative Medicine (2019) 19:292

DOI: 10.1186/s12906-019-2711-5

Human myometrium tissue was obtained from pregnant women undergoing a caesarean section and mounted in a myograph system. Tissue was allowed to contract spontaneously and *B. pinnatum* leaf press juice (BPJ), atosiban or nifedipine were added alone or in combination to the chamber. The effect of BPJ plus atosiban and BPJ plus nifedipine was compared to the effect of each substance alone, in terms of area under the curve (AUC), amplitude and frequency. To find out whether the effect observed was concentration dependent, substances alone or in combination were repeatedly added to the organ bath. Cell viability assays with human myometrial cell lines (hTERT-C3 and PHM1-41) were performed, for both combinations and substances alone.

My contributions to this publication: design of experiments, preparation of the myometrium tissue, performance of contractility experiments, cultivation of cells, design of cell experiments, recording and analysing data, writing the manuscript draft, and preparation of figures and tables.

Stefanie Lopes dos Santos

Santos et al. BMC Complementary and Alternative Medicine (2019) 19:292
https://doi.org/10.1186/s12906-019-2711-5

BMC Complementary and Alternative Medicine

RESEARCH ARTICLE Open Access

Bryophyllum pinnatum enhances the inhibitory effect of atosiban and nifedipine on human myometrial contractility: an in vitro study

S. Santos[1,2], C. Haslinger[1], M. Mennet[3], U. von Mandach[1], M. Hamburger[2] and A. P. Simões-Wüst[1*]

Abstract

Background: The herbal medicine *Bryophyllum pinnatum* has been used as a tocolytic agent in anthroposophic medicine and, recently, in conventional settings alone or as an add-on medication with tocolytic agents such as atosiban or nifedipine. We wanted to compare the inhibitory effect of atosiban and nifedipine on human myometrial contractility in vitro in the absence and in the presence of *B. pinnatum* press juice (BPJ).

Methods: Myometrium biopsies were collected during elective Caesarean sections. Myometrial strips were placed under tension into an organ bath and allowed to contract spontaneously. Test substances alone and at concentrations known to moderately affect contractility in this setup, or in combination, were added to the organ bath, and contractility was recorded throughout the experiments. Changes in the strength (measured as area under the curve (AUC) and amplitude) and frequency of contractions after the addition of all test substances were determined. Cell viability assays were performed with the human myometrium hTERT-C3 and PHM1–41 cell lines.

Results: BPJ (2.5 μg/mL), atosiban (0.27 μg/mL), and nifedipine (3 ng/mL), moderately reduced the strength of spontaneous myometrium contractions. When BPJ was added together with atosiban or nifedipine, inhibition of contraction strength was significantly higher than with the tocolytics alone ($p = 0.03$ and $p < 0.001$, respectively). In the case of AUC, BPJ plus atosiban promoted a decrease to 48.8 ± 6.3% of initial, whereas BPJ and atosiban alone lowered it to 70.9 ± 4.7% and to 80.9 ± 4.1% of initial, respectively. Also in the case of AUC, BPJ plus nifedipine promoted a decrease to 39.9 ± 4.6% of initial, at the same time that BPJ and nifedipine alone lowered it to 78.9 ± 3.8% and 71.0 ± 3.4% of initial. Amplitude data supported those AUC data. The inhibitory effects of BPJ plus atosiban and of BPJ plus nifedipine on contractions strength were concentration-dependent. None of the test substances, alone or in combination, decreased myometrial cell viability.

Conclusions: BPJ enhances the inhibitory effect of atosiban and nifedipine on the strength of myometrial contractions, without affecting myometrium tissue or cell viability. The combination treatment of BPJ with atosiban or nifedipine has therapeutic potential.

Keywords: *Bryophyllum pinnatum*, Atosiban, Nifedipine, Preterm, Myometrium, Contractility

* Correspondence: anapaula.simoes-wuest@usz.ch
[1]Department of Obstetrics, University Hospital Zurich, Schmelzbergstrasse 12/PF 125, 8091 Zurich, Switzerland
Full list of author information is available at the end of the article

Background

Preterm birth, defined as birth before 37 weeks of pregnancy, affects 5 to 18% of pregnancies. It is the number one cause of neonatal deaths, and the second leading cause of childhood death under 5 years of age [1–3]. Delaying preterm delivery frequently involves pharmacological inhibition of myometrial contractions (tocolysis) [4]. The main aim of a tocolytic treatment is to delay delivery long enough (24–48 h) for corticosteroid administration to the mother to achieve foetal lung maturation, and for transfer of the mother to a perinatal centre [4, 5]. Several types of tocolytics are currently in use [4, 6, 7]. Atosiban, a competitive oxytocin-vasopressin antagonist [8], and nifedipine, a calcium channel-blocking agent [9], are often given as first tocolytic treatment since they have good efficacy to side-effect ratios [10, 11]. A recent randomised study showed that atosiban and nifedipine resulted in similar perinatal outcomes [12]. Long term (> 1 week) tocolysis is seldom pursued and rarely achieved even though pregnancy prolongation favours perinatal outcomes [13].

Bryophyllum pinnatum (Lam.) Oken [syn. *Kalanchoe pinnata* (Lam.) Pers.; family Crassulaceae] is a perennial succulent plant that grows widely in tropical and subtropical areas. In Europe, *B. pinnatum* started to be used in anthroposophic medicine [14] as treatment of preterm labour [15, 16]. Retrospective analyses of the clinical practice revealed good efficacy of *B. pinnatum* preparations in this indication [15, 16], which was corroborated by a matched pair study [17]. Results of a prospective randomised trial on acute tocolysis are in line with good efficacy, but have to be interpreted with caution since - due to poor recruitment rate - the study was discontinued before completion [18]. All clinical studies demonstrated a very good tolerability of *B. pinnatum*. The use of such preparations in the treatment of pre-term contractions was supported by in vitro studies [19, 20].

An assessment of the internal treatment recommendations in the main Swiss obstetrics centres showed that *B. pinnatum* preparations are being prescribed for the treatment of preterm contractions [21]. This is in line with a Swiss online survey which showed that in approximately ¾ of the cases *B. pinnatum* preparations are administered in combination with synthetic tocolytics [22]. Comparable results were obtained in a retrospective analysis of the clinical practice at the University Hospital Zurich [23]. It is still not clear how *B. pinnatum* preparations influence the effects of tocolytics on myometrial contractility. We here compare the inhibitory effect of atosiban and nifedipine on human myometrial contractility in vitro in the absence and in the presence of *B. pinnatum* press juice (BPJ). Given a potentially synergistic effect of these substances, the question is of clinical interest.

Methods

Test substances

B. pinnatum leaves were harvested on the 25 March 2014 from *B. pinnatum* plants cultivated at the Medical Plants Garden located in S. Roque, Brazil, and that descend from seedlings brought from Weleda AG, Arlesheim, Switzerland, in the past. Plant collection did not affect Brazilian biodiversity and was done in accordance to Brazilian Environmental and Biodiversity laws, mainly Provisional Measure 2186–16 from 23 August 2001 that rules access to genetic resources and traditional knowledge. The Medical Plants Garden from S. Roque belongs to Weleda Brazil and the harvested *B. pinnatum* plants were identified by the Weleda employees Moacyr Copani and Paulo Copani. A voucher specimen ZSS 29717 was deposited at the Zurich Succulent Plant Collection. Leaves were sent by airmail to Weleda Arlesheim, Switzerland, in a refrigerated box. BPJ was obtained by mechanical pressing in a roller, the procedure used in the first step of the production of the active ingredient of Weleda *Bryophyllum* 50% chewable tablets (Weleda AG, Arlesheim). Unfiltered press juice was kept at − 80 °C until use.

Atosiban (Tractocile®, 7.5 mg/mL injectable solution), was purchased from Ferring Pharmaceuticals, Baar, Switzerland. Nifedipine was obtained from Sigma-Aldrich (purity ≥98%, N7634-1G); a 3.7 μg/mL stock solution was prepared in DMSO.

Design

The ethics committee of canton Zurich approved the study with human myometrium biopsies (KEK-ZH-Nr. 2014–0717, approval date 12.05.2015). Patients were asked prior to elective caesarean sections to donate a myometrium biopsy if the following inclusion criteria were fulfilled: single pregnancy, planned first caesarean section, negative HIV test, age > 18 years, and no tocolysis within 2 weeks before caesarean section.

A myometrial biopsy of approx. 5 g was taken from each study participant at the cranial margin of the uterotomy. The myometrial biopsy was immediately stored in Ringer solution and transported to the lab. Longitudinal strips of muscle of about 15 × 2 × 1 mm were cut and mounted in a myograph bath chamber. Each of the four myograph chambers contained 6 mL of Krebs solution (in mM: NaCl 118, $NaHCO_3$ 24.9, KCl 4.7, KH_2PO_4 1.24, $CaCl_2$ 2.48, $MgSO_4$ 1.21, Glucose 10, EDTA 0.034; pH = 7.4), with temperature regulated at 37 °C and bubbled with 95% O_2 and 5% CO_2 (PanGas, Dagmersellen, Switzerland). Contractions were recorded with a DMT800MS myograph (Danish Mayo Technology, Denmark) and transferred to a personal computer via a transducer (ADInstruments PowerLab 4/30). Myometrial strips were allowed to contract spontaneously (which took in most cases approximately 2 h). During this time, the Krebs solution was replaced every 30 min.

In preliminary experiments, concentrations of atosiban, nifedipine and BPJ were determined that would lead to moderate lowering (by 20–30%) of contraction strength. These concentrations were used in the main combination experiments, performed as described below. In preliminary experiments, two slightly different experimental protocols appeared equally promising. We used both protocols, one for the experiments with atosiban, and the second for nifedipine, to find out whether one of the protocols would result in lower standard error of the means (SEM) values.

Effect of the combination of BPJ with atosiban or with nifedipine on myometrial contractility

In all cases, regular spontaneous myometrial contractions in amplitude and frequency were recorded for 30 min.

When the effects of the combination of BPJ with atosiban were being studied, each one of four strips was treated with one test substance, and contractility was recorded for additional 30 min. Test substances were: Krebs solution, 5 μL (control; $n = 11$); BPJ, 15 μL (0.25% final concentration, corresponding to 2.5 μg/mL; $n = 13$); atosiban, 4.3 μL of 375 μg/mL (0.27 μg/mL final concentration; $n = 11$); and BPJ and atosiban combined (same concentrations, $n = 12$). Temporal and vehicle controls were run in parallel in each experiment to access the decay in contractility of the myometrium with time.

To study the combination of BPJ with nifedipine, Krebs solution, 5 μL (control; n = 13) or nifedipine, 5 μL of 3.7 μg/mL (final concentration 3 ng/mL; n = 11) was added to two chambers each, contractility was recorded for 30 min and thereafter 15 μL of BPJ (final concentration of 0.25 μg/mL; $n = 10$) was added to all four chambers. This resulted in two chambers with BPJ alone and two chambers with the combination of nifedipine with BPJ. After BPJ addition, contractions were recorded for 30 min (Fig. 1b).

Dose-dependency effect of combination treatments on myometrial contractility

To find out whether the effect of the combination treatments would further increase at higher concentrations, a previously described approach was followed [24]. In brief, when spontaneous contractions were regular for 20 min, Krebs solution was added (addition 0), and contractility was recorded for 20 min. Then, each strip was treated with one test substance by adding 4 times, at time intervals of 20 min, the same volume of a stock solution. Test solutions included: control, 5 μL Krebs solution; BPJ, 15 μL; combination of BPJ (15 μL) plus atosiban (4.3 μL of 375 μg/mL) or BPJ (15 μL) plus nifedipine (5 μL of 3.7 μg/mL). For each substance tested, 5 different biopsies were used ($n = 5$).

Vitality of myometrial strips

The exposure to the different test substances was followed by a 30-min washing period where Krebs solution was changed several times (at 5, 10, 20 and 30 min). Vitality of the strips was determined at the end of the experiment (30 min after washing) by observation of spontaneous contractions. In all cases, strips were contracting and data were included in the present analysis.

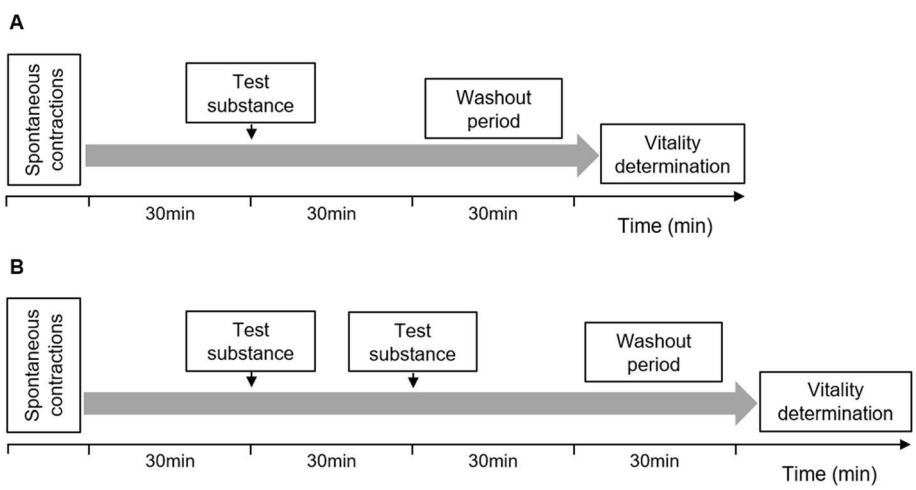


Fig. 1 Experimental design for measurement of myometrial contractions. Test substances were added to the organ bath when myometrium strips had been contracting regularly for 30 min. When the effects of BPJ and/or atosiban were being studied (**a**), Krebs solution (control), BPJ, atosiban, or the combination of BPJ and atosiban were added, and contractility was recorded for 30 min. When the effects of BPJ and/or nifedipine were being studied (**b**), Krebs solution (control; two strips) or nifedipine (two strips) was added, contractility was recorded for 30 min, and then BPJ was added to all chambers. Exposure to test substances was followed by a 30 min washout step, with change of Krebs solution at 5, 10, 20 and 30 min

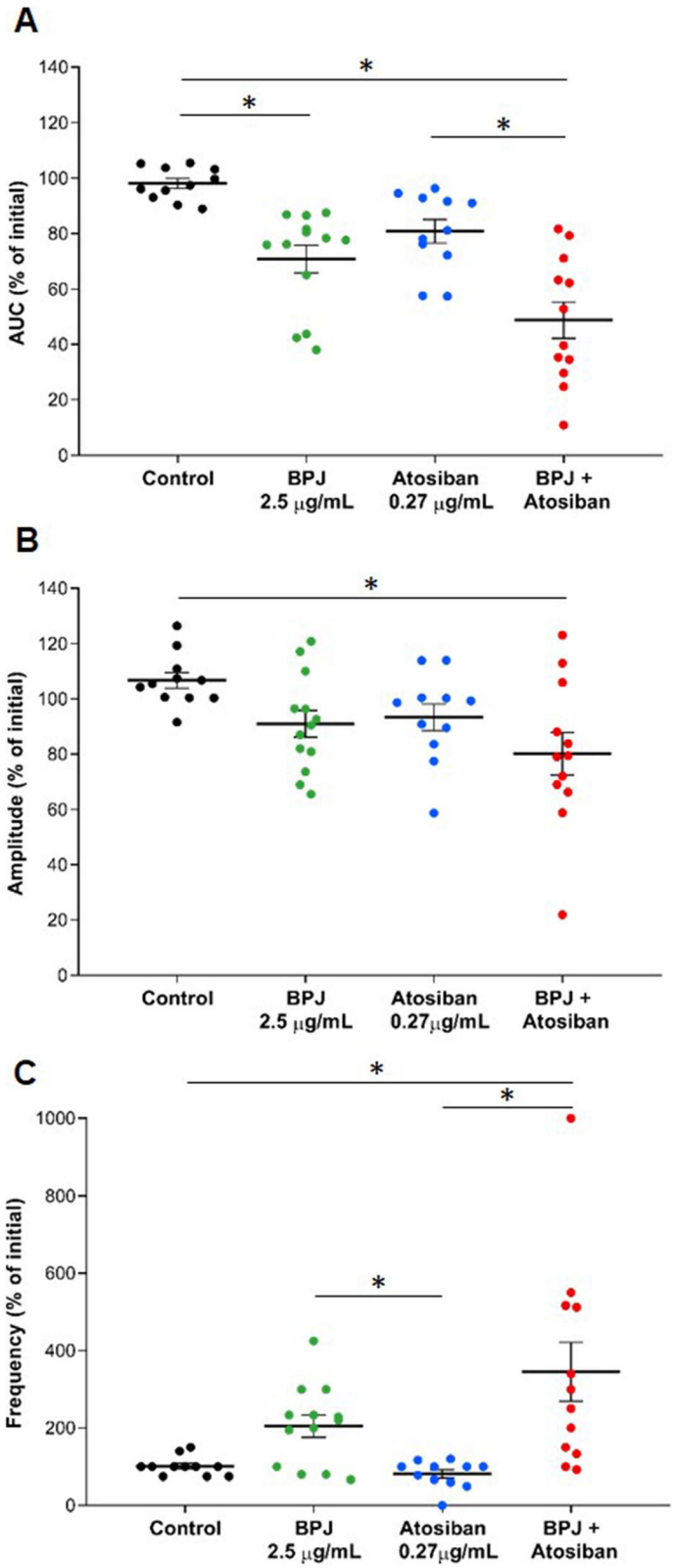


Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 Effect of BPJ, atosiban, and the combination of BPJ with atosiban on human myometrial contractility in vitro. BPJ (green; 15 μL), atosiban (blue; 4.3 μL of 375 μg/mL) or their combination (red, same concentrations) were added to the myograph chamber. The scatter dot plot shows the AUC (**a**), the amplitude (**b**), and the frequency (**c**) of contractions expressed as percentage of initial. Krebs solution was used as negative control (black, 5 μL). Data were obtained from 11 to 15 different biopsies ($n = 11–15$) and are presented as mean value ± SEM. *$p < 0.05$

Myograph data processing

Myometrium contractions were recorded by LabChart Pro 8.0.6 (ADInstruments, Germany) and analysed with the peak analysis module. For each contraction, the area under the curve (AUC) and the amplitude were analysed. Depending on the type of experiments, for each 20 or 30 min interval, the average AUC and average amplitude were calculated, and the number of contractions was noted (frequency). Initial values of AUC, amplitude and frequency of spontaneous contractions (before any addition) were set at 100%. Effects after addition of test substances were expressed as percentage of initial. When studying the combination of BPJ and atosiban and in all dose-dependency experiments, the values obtained in one strip per biopsy were used for further statistical analyses. When investigating the combination of BPJ and nifedipine, two strips per biopsy were used to determine the effect of each substance (BPJ, nifedipine, BPJ plus nifedipine, and control). In this case, average values of the two determinations were calculated and used for further statistical analyses.

Viability assays in myometrium cell lines

Human myometrial telomerase reverse transcriptase cell line (hTERT-C3) [25, 26], provided by M. Grãos (University of Coimbra, Portugal), was cultured in an 1:1 mixture of DMEM and F-12 supplemented with antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) and 10% (v/v) heat-inactivated foetal bovine serum (FBS) (all from Gibco, Paisley, UK). Human uterine myometrium smooth muscle cells (PHM1–41), obtained from American Type Culture Collection (ATCC® CRL-3046™) were maintained in ATCC-formulated DMEM (ATCC® No. 30–2002) supplemented with 0.1 mg/mL G-418 (Carl Roth, Zurich, Switzerland), 2 mM glutamine and 10% (v/v) heat-inactivated FBS.

hTERT-C3 cells were seeded at a density of 5×10^4 cells/mL (5×10^3 cells per well) and PHM1–41 cells at a density of 8×10^4 cells/mL (8×10^3 cells per well) into transparent 96-well microplates. 1 day after seeding, cells were exposed to BPJ (2.5–10.0 μg/mL), atosiban (0.27–1.08 μg/mL), nifedipine (3.0–12.0 ng/mL) or the combinations BPJ plus atosiban or BPJ plus nifedipine for 24 h. After exposure, resazurin (Alamar Blue, Invitrogen, Illkirch Cedex, France) was added to cells (final concentration 1.0 mg/mL), and the plate incubated at 37 °C for 4 h. The extent of resazurin reduction was measured in a microplate reader (SpectraMax Paradigm, Molecular Devices, Berkshire, UK) at 570 and 600 nm. For each substance tested, 4 independent experiments were carried out in quadruplicate. Ethyl methanesulfonate (30 mM) [27] and Triton X-100 (1%) were used as a positive control. In each experiment, wells with no test substance added to the culture medium served as untreated control (100% viability). Cell viability was determined according to the following equation:

$$\text{Viability} = \frac{(A_{570}-A_{600})\text{sample}-(A_{570}-A_{600})\text{blank}}{(A_{570}-A_{600})\text{control}-(A_{570}-A_{600})\text{blank}}$$

Cell morphology analysis

Myometrium hTERT-C3 cells and PHM1–41 cells were stained with fluorescent probes for nuclei (double-stranded DNA) and cytoplasm (F-actin), as follows. After treatment with test substances, alone or combined, for 24 h, cells were washed with phosphate buffered saline (PBS; Gibco, Paisley, UK) and fixed with 4% paraformaldehyde (PFA; from Artechemis, Zoffingen, Switzerland) in PBS for 20 min. Cells were then permeabilised with 0.3% Triton X-100 (Sigma, St. Louis, USA) in 1% bovine serum albumin (BSA; Sigma, St. Louis, USA) for 30 min. Then, cells were incubated with a 1:10000 dilution of 4′, 6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, USA) and 1:400 rhodamine phalloidin (Invitrogen, Illkirch Cedex, France) prepared in 0.1% Triton X-100 (Sigma, St. Louis, USA) in 1% BSA, for 4 h, in the dark. Cells were rinsed with PBS and examined with the Leica CTR 6000 microscope (Leica microsystems, Heerbrugg, Switzerland). The entire procedure was performed at room temperature.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., CA, USA). In all cases, a significance level of $p < 0.05$ was considered statistically significant.

Because of the slightly different experimental set-ups used to investigate the combinations of BPJ with atosiban and of BPJ with nifedipine in the myograph model, different tests were used in the two cases. Data from the combination of BPJ and atosiban measurements were analysed with the Kruskal Wallis test followed by Dunn's multiple comparisons test. Data from the combination of BPJ and nifedipine were analysed with the Wilcoxon test to compare control with BPJ and nifedipine with the combination (determinations in the same strips, paired

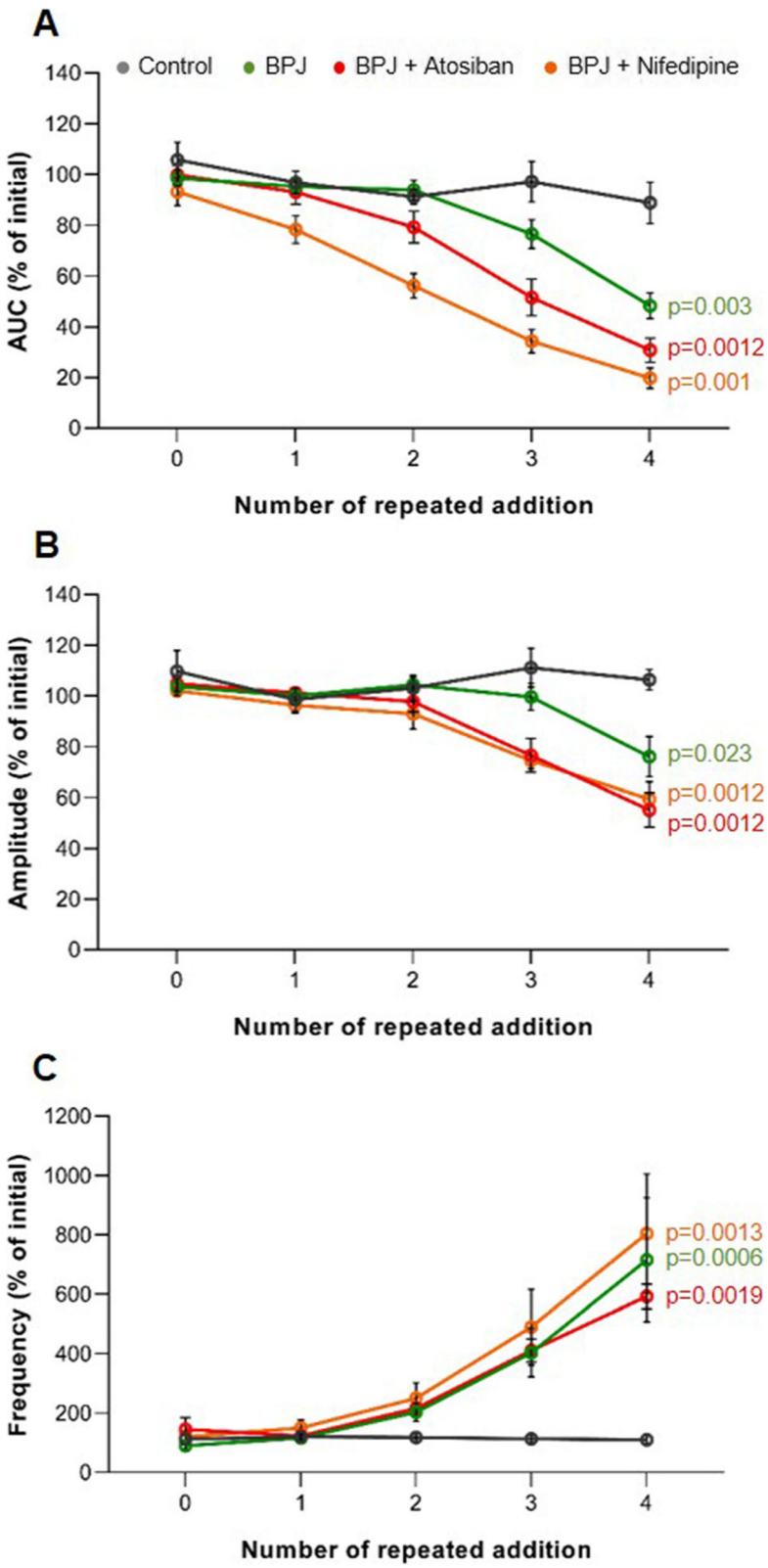


Fig. 3 (See legend on next page.)

(See figure on previous page.)
Fig. 3 Effect of repeated addition of BPJ plus atosiban (15 μL and 4.3 μL of 375 μg/mL, respectively) and of BPJ plus nifedipine (15 μL and 5 μL of 3.7 μg/mL, respectively) on human myometrial contractility in vitro. All test substances were repeatedly added to the myograph chamber. The line chart shows the AUC (**a**), the amplitude (**b**), and the frequency (**c**). Data were obtained with 5 different biopsies ($n = 5$) and are expressed as percentage of initial. The repeated addition of BPJ was performed for comparison; Krebs solution (5 μL) was used as control. *$p < 0.05$

test), and with the Mann-Whitney to compare control with nifedipine and with combination (determinations in different strips, unpaired test). Effects of combination treatments on myometrial contractility are expressed in scatter dot plots as mean values ± SEM.

For each test substance, dose-dependency data obtained in the myograph model and cell viability data was analysed with the paired, non-parametric Friedman test. Statistical analyses of cell viability data was followed by Dunn's multiple comparisons test. In the case of the single concentrations of positive controls used in cell viability assays, the paired t-test was used to compare their effects with untreated control. Myograph measurements on dose-dependency and cell viability data are given as mean ± SEM.

Results

Effect of BPJ and atosiban on human myometrial contractility

The exposure of contracting strips to 2.5 μg/mL BPJ and 0.27 μg/mL atosiban (final concentrations in the bath) led to a decrease of contraction strength (AUC and amplitude; Fig. 2a and b). BPJ alone lowered the AUC to 70.9 ± 4.7% of initial, which was significantly different from the control ($p = 0.001$), while 0.27 μg/mL of atosiban lowered AUC to 80.9 ± 4.1% of initial. When the combination of BPJ and atosiban was added to the organ bath, the AUC decreased to 48.8 ± 6.3%, a value significantly different from control and atosiban alone ($p < 0.001$ and $p = 0.03$, respectively; Fig. 2a).

BPJ decreased the amplitude to 91.0 ± 4.6% of initial, and atosiban to 93.4 ± 4.6% of initial, but the decreases were not statistically significant. The combination of BPJ and atosiban led to a significantly stronger decrease of amplitude (80.1 ± 7.4% of initial, $p = 0.01$; Fig. 2b) relative to control.

The frequency of myometrial contractions increased with the addition of BPJ to 204.7 ± 27.8% of initial, which was significantly higher than with atosiban (81.0 ± 10.1% of initial; $p = 0.03$). Also, the combination of BPJ and atosiban led to a significant increase of frequency (345.4 ± 73.0% of initial) relative to control and atosiban alone ($p = 0.010$ and $p < 0.001$, respectively; Fig. 2c) that per se did not increase frequency.

Stepwise increase of BPJ and atosiban concentrations led to successive decreases of myometrial contractility strength (Fig. 3a and b).

Effect of BPJ and nifedipine on myometrial contractility

BPJ, nifedipine, or the combination of the two led to a significant decrease of contractions relative to control (Fig. 4a). BPJ alone lead to a decrease to 78.9 ± 3.8% of initial ($p = 0.003$), and nifedipine decreased the AUC to 71.0 ± 3.4% of initial ($p < 0.001$). The combination of nifedipine with BPJ had the strongest effect, as the AUC of contractions was lowered to 39.9 ± 4.6% of initial, which was significantly different from the effect of nifedipine alone ($p < 0.001$).

The amplitude of myometrial contractions decreased with BPJ (91.7 ± 4.7%), nifedipine (86.4 ± 4.4%), and the combination (65.4 ± 5.3%). Compared to control, the effect of nifedipine ($p < 0.001$) and the combination of BPJ with nifedipine ($p = 0.003$) was significant. The combination of BPJ and nifedipine also significantly decreased the amplitude when compared to nifedipine alone ($p = 0.002$; Fig. 4b).

As shown in Fig. 4c, BPJ strongly increased the frequency of myometrial contractions to 257.1 ± 40.6% of initial ($p < 0.001$). In contrast, nifedipine alone had no effect on frequency when compared to control. However, the combination of BPJ and nifedipine led to an increase of 190.1 ± 22.2% of initial, which was significantly different from control ($p < 0.0001$) and from nifedipine alone ($p < 0.001$; Fig. 4c).

Exposing the strips to successively higher concentrations of the combination of BPJ and nifedipine led to stepwise increases of the inhibitory effects on myometrial contractility (Fig. 3a and b, data on BPJ + Nifedipine).

Effects on myometrial viability

Under our experimental conditions, myometrium strips were still contracting spontaneously after the washing step at the end of the myograph experiments, revealing that the test substances (single or in combinations) were not toxic to myometrial tissue (data not shown). To assess the cytotoxicity of the test substances using a different read-out, viability experiments were performed with two human myometrial cell lines (hTERT-C3 and PHM1–41). The test substances, alone or in combination, were not cytotoxic at similar or even higher concentrations than those used in the main combinations experiment, and at a markedly longer exposure time (24 h; Fig. 5a). At the end of cell viability experiments, cell morphology was evaluated by fluorescence microscopy. Visual examination revealed that the test substances did not affect the morphology of myometrial cells. In

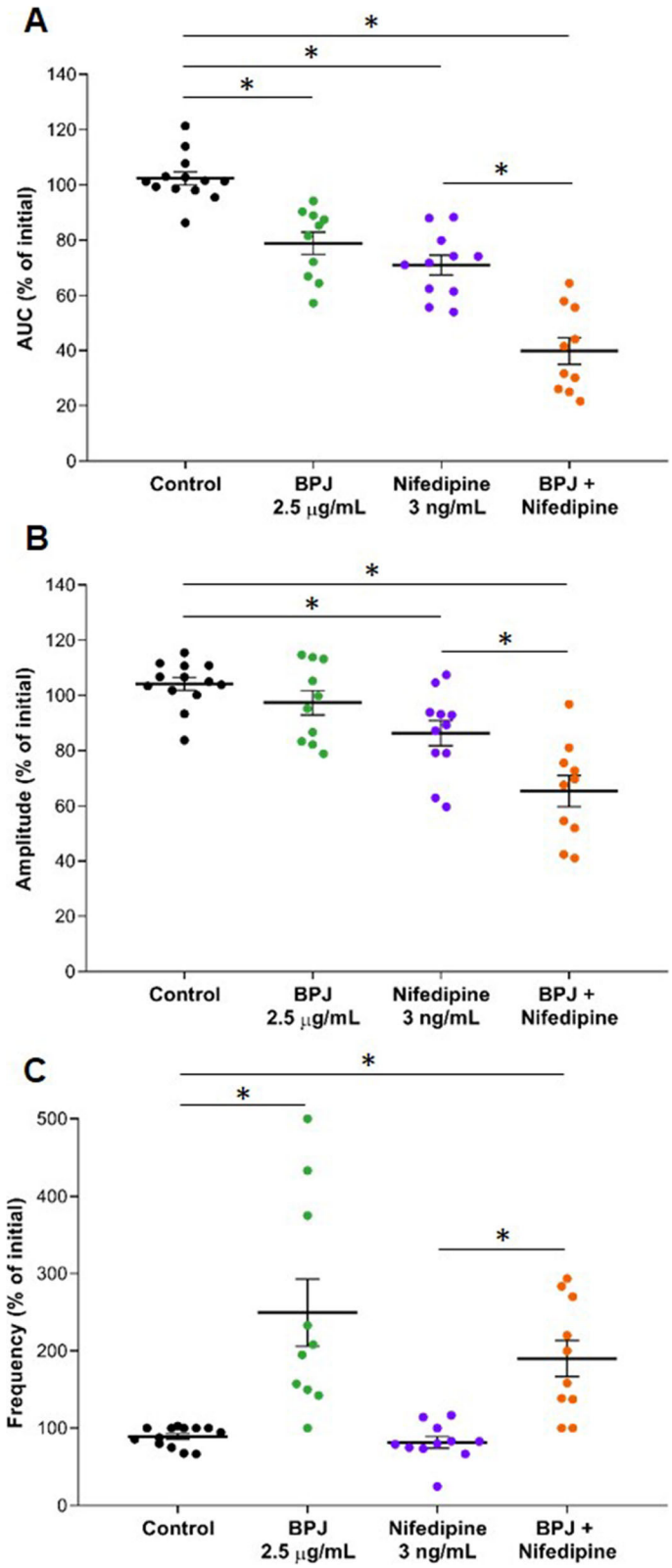


Fig. 4 (See legend on next page.)

(See figure on previous page.)

Fig. 4 Effect of BPJ, nifedipine, and the combination of BPJ with nifedipine on human myometrial contractility in vitro. BPJ (green; 15 μL), nifedipine (violet; 5 μL of 3.7 μg/mL), or their combination (orange, same concentrations) were added to the myograph chamber. The scatter dot plot shows the AUC (**a**), the amplitude (**b**), and the frequency (**c**) expressed as percentage of initial. Krebs solution was used as negative control (black, 5 μL). Data were obtained from 11 to 13 different biopsies (n = 11–13) and are presented as mean value ± SEM. *p < 0.05

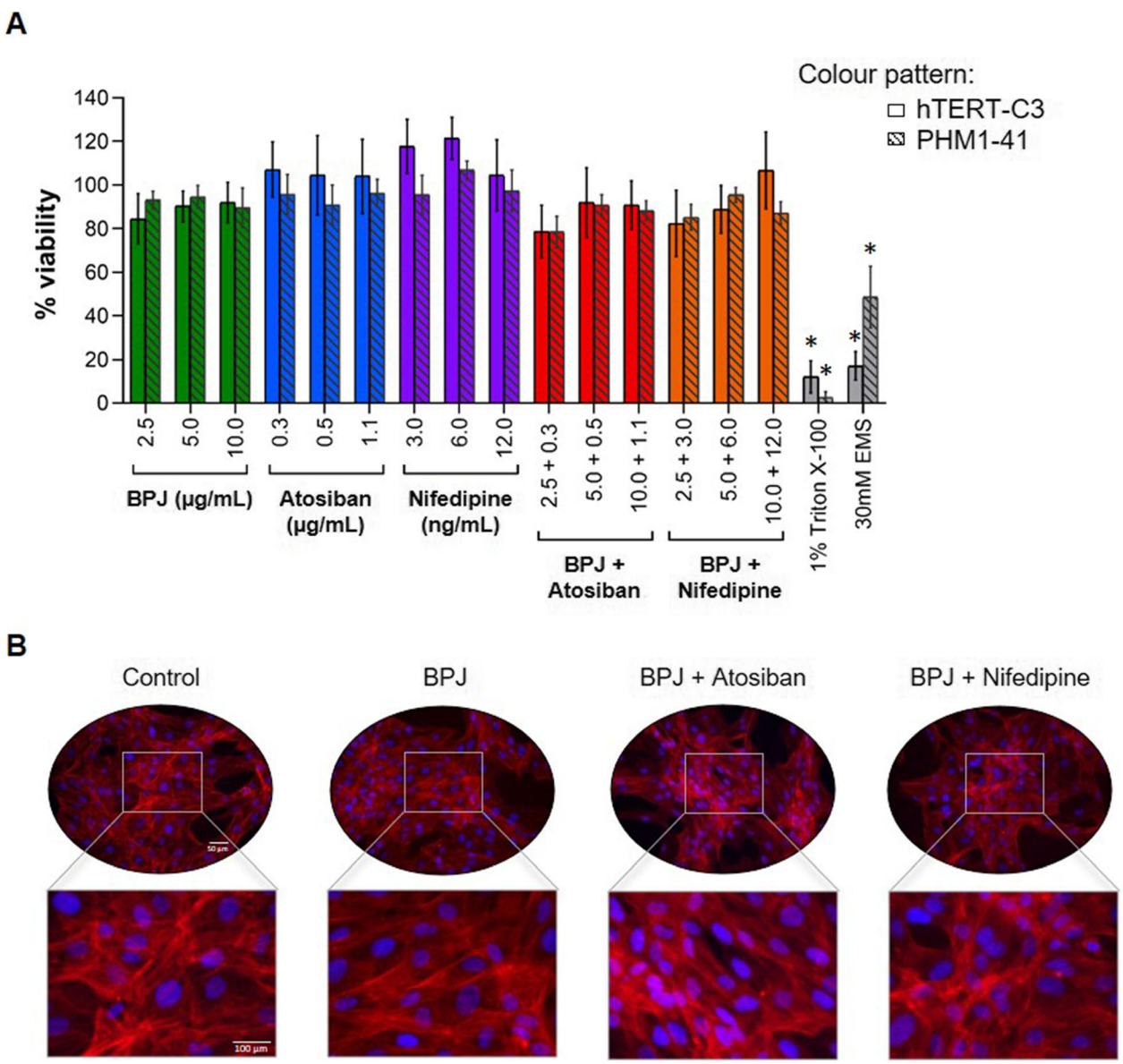


Fig. 5 Effect of BPJ, atosiban, nifedipine, BPJ plus atosiban and BPJ plus nifedipine on myometrium cell viability. (**a**) Cell viability assays were performed in the presence of BPJ (2.5–10.0 μg/mL), atosiban (0.3–1.1 μg/mL) and nifedipine (3.0–12.0 ng/mL), as well as of BPJ plus atosiban and BPJ plus nifedipine (same concentrations as with single treatments) using hTERT-C3 and PHM1–41 human myometrium cell lines. Cells were incubated with the test substances for 24 h. Triton X-100 (1%) and ethyl methanesulfonate (30 mM) were used as positive controls. Data is presented as mean ± SEM of 4 independent experiments ($n = 4$), each carried out in quadruplicate; *$p < 0.05$. (**b**) Staining of nuclei (blue) and actin (red) from hTERT-C3 cells untreated (i.e. control) or upon treatment with BPJ (10.0 μg/mL), BPJ plus atosiban (10.0 μg/mL and 1.1 μg/mL, respectively), and BPJ plus nifedipine (10.0 μg/mL and 12.0 ng/mL, respectively). The images are representative of four independent cultures

particular, the intact nucleus morphology of hTERT-C3 cells (Fig. 5b) or PHM1–41 cells (data not shown) treated with the highest concentrations of the various test substances reveals that the cells were not undergoing apoptosis.

Discussion

Press juice from *B. pinnatum* leaves, the active ingredient of chewable tablets that are being used in the management of preterm labour in Switzerland, enhances the inhibitory effect of the oxytocin receptor antagonist atosiban on contraction strength in human myometrium strips. BPJ also enhances the effect of nifedipine, a voltage-dependent calcium channel blocker that is in off-label use as a tocolytic. Both the combination BPJ with atosiban and BPJ with nifedipine show dose-dependent effects on human myometrial contractility. Reduced viability of myometrial tissue or cells does not play a role in the observed results. Taken together, our data corroborate the clinical use of these combination treatments.

The main strength of our study is the use of the physiologically most relevant model to study the process of myometrial contractions in human material, with the possibility to compare the effect of different substances, alone and in combination. In fact, our study depicts a proof of principle for the therapeutic potential of the combination of standard tocolytic medications with BPJ. Alternatives would be animal models, but the process of labour differs between humans and animal species [28, 29]. Limitations of our model are the availability of myometrium biopsies and the very low throughput that can be achieved with an organ bath model. For these reasons, the number of tested concentrations had to be kept low. Also due to the low throughput, it would be advantageous to further reduce the variability of results (and therefore of needed experiments) in future projects. For this reason, two variant experimental protocols were used in this work. The comparable SEM values obtained indicated that the two protocols were equally suited.

Several signalling pathways are known to increase intracellular calcium concentrations leading to contraction of myometrium cells. Some of these pathways are triggered by binding of oxytocin to the corresponding G-protein coupled receptor, but membrane depolarisation with concomitant opening of voltage-gated calcium channels may also play a role [30]. The two tocolytics used in the present study prevent the increase in intracellular calcium concentration by different mechanisms, namely by blocking oxytocin receptors in the case of atosiban, or by blocking voltage-gated calcium channels in case of nifedipine [11]. Previous work showed that BPJ inhibits oxytocin-induced increase of intracellular calcium concentration in myometrium cells. BPJ did not prevent, but delayed the depolarisation-induced increase of intracellular calcium in cells with voltage-gated channels [31]. In myometrial strips, the combination of atosiban with calcium-channel blockers (nicardipine or nifedipine) led to additive inhibitory effects, indicating that simultaneous targeting of these two pathways has clinical potential [32, 33]. Therefore, it appears conceivable that the delaying effect of BPJ on depolarisation-induced increase of intracellular calcium can enhance the effects of atosiban. Likewise, the inhibition of oxytocin-induced increase of intracellular calcium concentration by BPJ should synergise with the effects of nifedipine. To which extent inhibition by BPJ of each of these two signalling pathways contributes to the observed in vitro results or to the effectiveness of *B. pinnatum* preparations in the treatment of preterm labour is currently not known.

BPJ is known to increase the contraction frequency of myometrium strips [19, 20] and this was also observed in the combination with atosiban or nifedipine (Figs. 2, 3 and 4). Our previous work suggests that various components of *B. pinnatum* leaves might contribute to increase contraction frequency of myometrium strips [24]. Both a bufadienolide-enriched fraction and a flavonoid-enriched fraction (but not the corresponding flavonoid aglycon mixture) seemed to contribute to the frequency increase. Whether the signalling pathways activated by BPJ might trigger a partial membrane-depolarisation reserves further investigations. Trying to translate the increase in frequency seen in myometrium strips into the clinical situation, we feel tempted to suggest that *B. pinnatum* preparations – but not other tested tocolytics – could induce a type of conversion of labour-contractions into other, high frequency and painless, and most importantly not-effective, contractions. These are well known in the praxis, and often interpreted as myometrium training uterine contractions that do not lead to labour. In retrospective studies on the tolerability of *B. pinnatum*, no clinically relevant increases of contraction frequency have been observed, neither when used as single treatment nor in combination with tocolytics [15–17, 23]. Also, no increase was reported in a prospective observational study which even included women with uterine tachysystoles [22]. On the contrary, a significant lowering of contraction frequency was observed after 4 h of treatment with *B. pinnatum* 50% tablets in a previous randomised trial on acute tocolysis ($n = 13$) [18].

Although the main combination experiments were performed in vitro and with concentrations leading to moderate effects, we compared the concentrations of atosiban and nifedipine used in vitro with the corresponding plasma concentrations during tocolysis. In the case of atosiban, the mean plasma concentration at steady state is 0.44 ± 0.07 μg/mL [34], and in the case of nifedipine is 67.4 ± 28.4 ng/mL [35]. Therefore, whereas the concentration of atosiban in the myograph experiments is rather close to that measured in the plasma, the

concentration of nifedipine was markedly lower. Given the lack of pharmacokinetic data with BPJ, it is not possible at this point to compare the concentration in the myograph experiments with known plasma concentrations.

Currently used standard tocolytic treatments are not always able to prolong pregnancy for at least 48 h [7]. At the same time, combinations of standard tocolytics are not recommended by the Swiss Society for Gynaecology and Obstetrics current Swiss guidelines [36], nor by the guidelines from the National Institute for Health and Care Excellence (NICE) [37] because of concerns about side-effects. Our data show that BPJ enhances the inhibitory effect of atosiban and nifedipine on myometrium contractility. In Germany and Switzerland, *B. pinnatum* preparations (containing BJP as the active ingredient) have been used for decades in clinics and private practices of anthroposophic medicine [15, 16, 38]. In Switzerland, *B. pinnatum* preparations are being recommended [21] and used to stop pre-term contractions also in conventional clinical practice, often as an add-on treatment [22, 23]. In the case of atosiban, a combination at low dosages with *B. pinnatum* would lower the overall medication costs. As for nifedipine, a lowering of the dosage in a combination with *B. pinnatum* would have the advantage of limiting the well-known cardiovascular side effects, such as palpitations, hypotension, flushes, headache, and gastro-intestinal symptoms like gastric upset and constipation [39].

Conclusion

We provide here evidence for the potential of drug combinations of atosiban and nifedipine with *B. pinnatum*. Such combinations may lower the required dosage of tocolytics, thereby decreasing treatment costs and reducing maternal and foetal side effects. This could help to reduce early tocolysis failure and to increase the percentage of patients that reach a 48 h delay of delivery. Prospective randomised studies are needed to substantiate such combination treatments.

Abbreviations

AUC: Area under the curve; BPJ: *Bryophyllum pinnatum* leave press juice

Acknowledgements

The authors thank Alexandra Dolder for excellent technical support, and all professionals at the University Hospital Zürich, Department of Obstetrics, who facilitated the collection of myometrium biopsies. We are grateful to Dr. Niels Hagenbuch, Institute of Epidemiology, Biostatistics and Prevention, University of Zurich, for statistical support. We are indebted to all other members of the *Bryophyllum* Study-Group for stimulating discussions.

Author's contributions

SS, CH, UM and APSW designed the study. SS performed the experiments, analysed the data and wrote the first version of the manuscript, under the supervision of APSW. CH recruited the patients and organised biopsy collection. MH and MM were involved in the interpretation of data and provided critical revision of the manuscript. All authors were closely involved in revising the article and agreed with the final version.

Funding

Financial support was provided by Weleda AG and the Johannes Kreyenbühl Foundation. The sponsors had no influence on data collection, management or analysis of the data.

Availability of data and materials

Data obtained during the current study are available from the corresponding author on reasonable request. The biological materials used (myometrium strips) are not available since they have to be used fresh.

Ethics approval and consent to participate

This study was approved by the ethics committee of canton Zurich (KEK-ZH-Nr. 2014–0717, approval date 12.05.2015). Written informed consent was obtained the day before surgery.

Consent for publication

Not applicable.

Competing interests

MM is an employee of Weleda AG, the company that produces the preparations of *Bryophyllum pinnatum*. APSW received research funding from Weleda AG during the last 5 years.

Author details

[1]Department of Obstetrics, University Hospital Zurich, Schmelzbergstrasse 12/PF 125, 8091 Zurich, Switzerland. [2]Division of Pharmaceutical Biology, University of Basel, Basel, Switzerland. [3]Weleda AG, Arlesheim, Switzerland.

Received: 25 January 2019 Accepted: 10 October 2019
Published online: 04 November 2019

References

1. World Health Organization [http://www.who.int/mediacentre/factsheets/fs363/en/].
2. Blencowe H, Cousens S, Chou D, Oestergaard M, Say L, Moller A-B, Kinney M, Lawn J. Born too soon: the global epidemiology of 15 million preterm births. Reprod Health. 2013;10(1):S2.
3. Lawn JE, Davidge R, Paul VK, von Xylander S, de Graft JJ, Costello A, Kinney MV, Segre J, Molyneux L. Born too soon: care for the preterm baby. Reprod Health. 2013;10(1):S5.
4. Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes. Science. 2014;345(6198):760–5.
5. Arrowsmith S, Kendrick A, Wray S. Drugs acting on the pregnant uterus. Obstet Gynaecol Reprod Med. 2010;20(8):241–7.
6. Schleußner E. The prevention, diagnosis and treatment of premature labor. Dtsch Arztebl Int. 2013;110(13):227.
7. Haas DM, Imperiale TF, Kirkpatrick PR, Klein RW, Zollinger TW, Golichowski AM. Tocolytic therapy: a meta-analysis and decision analysis. Obstet Gynecol. 2009;113(3):585–94.
8. Flenady V, Reinebrant HE, Liley HG, Tambimuttu EG, Papatsonis DN. Oxytocin receptor antagonists for inhibiting preterm labour. Cochrane Database Syst Rev. 2014 Jun;6(6):CD004452.
9. Flenady V, Wojcieszek AM, Papatsonis DN, Stock OM, Murray L, Jardine LA, Carbonne B. Calcium channel blockers for inhibiting preterm labour and birth. Cochrane Database Syst Rev. 2014;6:1–34.
10. van Vliet EO, Schuit E, Heida KY, Opmeer BC, Kok M, Gyselaers W, Porath MM, Woiski M, Bax CJ, Bloemenkamp KW. Nifedipine versus atosiban in the treatment of threatened preterm labour (assessment of perinatal outcome after specific Tocolysis in early labour: APOSTEL III-trial). BMC Pregnancy Childbirth. 2014;14(1):93.
11. Haram K, Mortensen JHS, Morrison JC. Tocolysis for acute preterm labor: does anything work. J Matern Fetal Neonatal Med. 2015;28(4):371–8.
12. Van Vliet EO, Nijman TA, Schuit E, Heida KY, Opmeer BC, Kok M, Gyselaers W, Porath MM, Woiski M, Bax CJ. Nifedipine versus atosiban for threatened preterm birth (APOSTEL III): a multicentre, randomised controlled trial. Lancet. 2016;387(10033):2117–24.
13. Baschat AA, Cosmi E, Bilardo CM, Wolf H, Berg C, Rigano S, Germer U, Moyano D, Turan S, Hartung J. Predictors of neonatal outcome in early-onset placental dysfunction. Obstet Gynecol. 2007;109(2, Part 1):253–61.

14. Kienle GS, Albonico H-U, Baars E, Hamre HJ, Zimmermann P, Kiene H. Anthroposophic medicine: an integrative medical system originating in Europe. Global advances in health and medicine. 2013;2(6):20–31.
15. Fürer K, Simões-Wüst AP, von Mandach U, Hamburger M, Potterat O. *Bryophyllum pinnatum* and related species used in anthroposophic medicine: constituents, pharmacological activities, and clinical efficacy. Planta Med. 2016;82(11/12):930–41.
16. Hamburger M, Potterat O, Furer K, Simoes-Wüst AP, von Mandach U. *Bryophyllum pinnatum*-reverse engineering of an Anthroposophic herbal medicine. Nat Prod Commun. 2017;12(8):1359–64.
17. Plangger N, Rist L, Zimmermann R, von Mandach U. Intravenous tocolysis with *Bryophyllum pinnatum* is better tolerated than beta-agonist application. Eur J Obstet Gynecol Reprod Biol. 2006;124(2):168–72.
18. Simões-Wüst AP, Lapaire O, Hösli I, Wächter R, Fürer K, Schnelle M, Mennet-von Eiff M, Seifert B, von Mandach U. Two randomised clinical trials on the use of *Bryophyllum pinnatum* in pre-term labour – results after discontinuation. Complementary Medicine Research. 2018.
19. Gwehenberger B, Rist L, Huch R, von Mandach U. Effect of *Bryophyllum pinnatum* versus fenoterol on uterine contractility. Eur J Obstet Gynecol Reprod Biol. 2004;113(2):164–71.
20. Wächter R, Brenneisen R, Hamburger M, Mennet M, Schnelle M, Worel A, Simões-Wüst AP, Von Mandach U. Leaf press juice from *Bryophyllum pinnatum* (Lamarck) Oken induces myometrial relaxation. Phytomedicine. 2011;19(1):74–82.
21. Schenkel L, Simões-Wüst AP, Hoesli I, von Mandach U. Drugs in pregnancy and lactation - medications used in Swiss obstetrics. Z Geburtshilfe Neonatol. 2018;222:1–14.
22. Fürer K, Simões-Wüst AP, Winkler A, Amsler N, Schnelle M, von Mandach U. Die Anwendung von *Bryophyllum pinnatum*-Präparaten in der Geburtshilfe und Gynäkologie-eine multizentrische prospektive Beobachtungsstudie. Complementary Medicine Research. 2015;22(4):231–6.
23. Simões-Wüst AP, Nietlispach A, Mennet M, von Mandach U. *Bryophyllum pinnatum* use in tocolysis in a conventional setting: a retrospective analysis from obstetric clinical practice. BMC Complement Altern Med. 2017; 17(Suppl 1-333):136.
24. Santos S, Haslinger C, Klaic K, Faleschini MT, Mennet M, Potterat O, von Mandach U, Hamburger M, APJPm S-W. A Bufadienolide-Enriched Fraction of Bryophyllum pinnatum Inhibits Human Myometrial Contractility In Vitro. Planta Med. 2019;85:385–93.
25. Condon J, Yin S, Mayhew B, Word RA, Wright W, Shay J, Rainey WE. Telomerase immortalization of human myometrial cells. Biol Reprod. 2002; 67(2):506–14.
26. Devost D, Zingg HH. Novel in vitro system for functional assessment of oxytocin action. Am J Physiol-Endocrinol Metab. 2007;292(1):E1–6.
27. Ray SD, Fariss MW. Role of cellular energy status in tocopheryl hemisuccinate cytoprotection against ethyl methanesulfonate-induced toxicity. Arch Biochem Biophys. 1994;311(1):180–90.
28. Smith R. Parturition. N Engl J Med. 2007;356(3):271–83.
29. Arthur P, Taggart M, Mitchell B. Oxytocin and parturition: a role for increased myometrial calcium and calcium sensitization? Front Biosci. 2007;12:619.
30. Arrowsmith S, Wray S. Oxytocin: its mechanism of action and receptor signalling in the myometrium. J Neuroendocrinol. 2014;26(6):356–69.
31. Simões-Wüst AP, Grãos M, Duarte C, Brenneisen R, Hamburger M, Mennet M, Ramos M, Schnelle M, Wächter R, Worel A. Juice of *Bryophyllum pinnatum* (lam.) inhibits oxytocin-induced increase of the intracellular calcium concentration in human myometrial cells. Phytomedicine. 2010; 17(12):980–6.
32. Doret M, Mellier G, Gaucherand P, Saade GR, Benchaib M, Frutoso J, Pasquier JC. The *in vitro* effect of dual combinations of ritodrine, nicardipine and atosiban on contractility of pregnant rat myometrium. BJOG. 2003; 110(8):731–4.
33. Kuć P, Laudański P, Pierzyński P, Laudański T. The effect of combined tocolysis on *in vitro* uterine contractility in preterm labour. Adv Med Sci. 2011;56(1):88–94.
34. Goodwin TM, Millar L, North L, Abrams LS, Weglein RC, Holland ML. The pharmacokinetics of the oxytocin antagonist atosiban in pregnant women with preterm uterine contractions. Am J Obstet Gynecol. 1995;173(3):913–7.
35. Papatsonis DN, Bos JM, van Geijn HP, Lok CA, Dekker GA. Nifedipine pharmacokinetics and plasma levels in the management of preterm labor. Am J Ther. 2007;14(4):346–50.
36. Hösli I, Sperschneider C, Drack G, Zimmermann R, Surbek D, Irion O. Tocolysis for preterm labor: expert opinion. Arch Gynecol Obstet. 2014; 289(4):903–9.
37. National Institute for Health and Care Excellence (NICE): Preterm labour and birth. In. NICE: National Institute for Health and Care Excellence; 2015.
38. Simões-Wüst AP, Jeschke E, Mennet M, Schnelle M, Matthes H, von Mandach U. Prescribing pattern of *Bryophyllum* preparations among a network of anthroposophic physicians. \ 2012; 19(6):293–301.
39. Al-Omari WR, Al-Shammaa HB, Al-Tikriti EM, Ahmed KW. Atosiban and nifedipine in acute tocolysis: a comparative study. Eur J Obstet Gynecol Reprod Biol. 2006;128(1):129–34.

Publisher's Note

2.2. A bufadienolide-enriched fraction of *Bryophyllum pinnatum* inhibits human myometrial contractility

Stefanie Santos, Christian Haslinger, Kristian Klaic, Maria T. Faleschini, Mónica Mennet, Olivier Potterat, Ursula von Mandach, Matthias Hamburger, Ana Paula Simões-Wüst

Planta Medica (2019) 85:385-393

DOI: 10.1055/a-0810-7704

Human myometrium tissue was obtained from pregnant women undergoing a caesarean section and mounted in a myograph system. Tissue was allowed to contract spontaneously and a flavonoid-enriched fraction (FEF), the corresponding flavonoid aglycon mixture (A-Mix), a bufadienolide-enriched fraction (BEF), *B. pinnatum* leaf press juice (BPJ), and nifedipine were added repeatedly to the myograph chamber. Effect of each test substance was compared to the effect of a non-stimulated strip (control), in terms of AUC, amplitude and frequency. Vitality of the strips was determined at the end of the experiment by observation of spontaneous contractions. Cell viability assays with human myometrial cell lines (hTERT-C3 and PHM1-41) were performed, for FEF, A-Mix, BEF, and BPJ.

My contributions to this publication: design and performance of tissue experiments, preparation of the myometrium tissue, performance of contractility experiments, cultivation of cells, design of cell experiments, help master student perform cell experiments, recording and analysing data, writing the manuscript draft, and preparation of figures and tables.

Stefanie Lopes dos Santos

A Bufadienolide-Enriched Fraction of *Bryophyllum pinnatum* Inhibits Human Myometrial Contractility *In Vitro*

Authors

Stefanie Santos[1,2], Christian Haslinger[1], Kristian Klaic[1], Maria T. Faleschini[2], Mónica Mennet[3], Olivier Potterat[2], Ursula von Mandach[1], Matthias Hamburger[2], Ana Paula Simões-Wüst[1]

Affiliations

1 Department of Obstetrics, University Hospital Zurich, Zurich, Switzerland
2 Division of Pharmaceutical Biology, University of Basel, Basel, Switzerland
3 Weleda AG, Arlesheim, Switzerland

Key words

Bryophyllum pinnatum, Crassulaceae, myometrium, flavonoids, bufadienolides, toxicity

received August 20, 2018
revised November 6, 2018
accepted November 27, 2018

Bibliography

DOI https://doi.org/10.1055/a-0810-7704
Published online December 18, 2018 | Planta Med 2019; 85: 385–393 © Georg Thieme Verlag KG Stuttgart · New York | ISSN 0032-0943

Correspondence

PD Dr. Ana Paula Simões-Wüst
University Hospital of Zurich, Department of Obstetrics
Schmelzbergstrasse 12/PF 125, Path G51a, 8091 Zürich, Switzerland
Phone: + 41 44 2 55 51 31, Fax: + 41 44 2 55 44 30
anapaula.simoes-wuest@usz.ch

Supporting information available online at http://www.thieme-connect.de/products

ABSTRACT

Bryophyllum pinnatum has been used since the 1970s to prevent premature labour, first in anthroposophic hospitals and, more recently, also in the main Swiss perinatal centres. However, it is not known which compounds in *B. pinnatum* leaves contribute to the tocolytic effect. Here we studied the effects of a flavonoid-enriched fraction, the corresponding flavonoid aglycon mixture, a bufadienolide-enriched fraction, and *B. pinnatum* leaf press juice on human myometrial contractility *in vitro*. The strength (area under the curve and amplitude) and frequency of contractions were recorded using strips of human myometrium mounted in an organ bath system. Cell viability assays were performed with the human myometrium hTERT-C3 and PHM1–41 cell lines. Repeated addition of the flavonoid-enriched fraction, flavonoid aglycon mixture, bufadienolide-enriched fraction, or *B. pinnatum* leaf press juice led to a progressive decrease of contraction strength, without jeopardising the vitality of myometrium strips. The bufadienolide-enriched fraction was the most active, since 1 µg/mL of the bufadienolide-enriched fraction lowered the area under the curve to 40.1 ± 11.8 % of the initial value, whereas 150 µg/mL of the flavonoid-enriched fraction, 6.2 µg/mL of the flavonoid aglycon mixture, and 10 µg/mL of the *B. pinnatum* leaf press juice were required to achieve comparable inhibition. A progressive increase of contraction frequency was observed, except in the case of the flavonoid aglycon mixture, which did not affect frequency. None of the test substances decreased myometrial cell viability, even at concentrations of 500 µg/mL of the flavonoid-enriched fraction, 40 µg/mL of the flavonoid aglycon mixture, 3.8 µg/mL of the bufadienolide-enriched fraction, and 75 µg/mL of the *B. pinnatum* leaf press juice, i.e., higher than those used in the myometrium experiments. Given the concentrations of flavonoids in the flavonoid-enriched fraction and *B. pinnatum* leaf press juice, and of bufadienolides in the bufadienolide-enriched fraction and *B. pinnatum* leaf press juice, it appears that bufadienolides may be mainly responsible for the relaxant effect.

ABBREVIATIONS

A-Mix	flavonoid aglycon mix
AUC	area under the curve
BEF	bufadienolide-enriched fraction
BPJ	*Bryophyllum pinnatum* leaf press juice
FEF	flavonoid-enriched fraction
PC	positive control

Introduction

Bryophyllum pinnatum (Lam.) Oken [syn. *Kalanchoe pinnata* (Lam.) Pers.; family Crassulaceae] is a succulent perennial plant native to Madagascar that now grows widely in tropical and subtropical regions around the globe. In ethnomedicine, *B. pinnatum* has multiple uses including the treatment of wounds, diabetes mellitus, joint pain, headache, hypertension, and kidney stones, and nausea and vomiting in cancer patients. For *B. pinnatum* extracts, antifungal, antimicrobial, anti-inflammatory, and analgesic properties have been reported [1, 2].

In Europe, the use of remedies prepared from *B. pinnatum* leaves was limited for a long time to anthroposophic medicine. Initially, they were used for the treatment of various hyperactivity disorders [3, 4], and only in the 1970s was *B. pinnatum* introduced by the German gynaecologist Werner Hassauer (1928–1993) as a routine treatment of preterm labour [5]. In Switzerland, *B. pinnatum* is used in gynaecology and obstetrics against premature contractions, restlessness, and overactive bladder [6]. A recent assessment of the internal treatment recommendations in the main Swiss obstetrics centres revealed that *B. pinnatum* preparations are suggested for the treatment of preterm contractions, as well as anxiety, restlessness, and sleep disorders [7]. Several clinical studies documented a very good tolerability of *B. pinnatum* preparations when used for these indications [3, 4, 6, 8–12]. Moreover, pharmacovigilance data for the *Bryophyllum* preparation used in Switzerland (*B. pinnatum* 50% tablets) and for a comparable preparation in use in Germany and France (*B. pinnatum* 50% powder) corroborate the very good tolerability (Weleda AG, pharmacovigilance data).

Preterm labour and prematurity (i.e., birth before 37 weeks of pregnancy) are the main determinants of perinatal mortality and long-term morbidity [13, 14]. Since preterm uterine contractions correlate highly with preterm birth, their inhibition by tocolytics (beta-adrenergic receptor agonists, calcium channel blockers, or oxytocin receptor antagonists) constitute a major treatment element. Most studies have shown that administration of tocolytics may delay pregnancy for 48 h with the goal to administer antenatal corticosteroid therapy for foetal lung maturation and to allow intrauterine transfer to a tertiary care perinatal centre [15, 16]. However, tocolysis was not shown to prevent preterm birth from occurring or to reduce neonatal morbidity or mortality [17, 18]. Therefore, additional therapeutic options are needed. As a tocolytic agent, *B. pinnatum* was shown to be effective and to lead to significantly less maternal adverse effects than beta-adrenergic receptor agonists [10]. *In vitro* studies showed that *B. pinnatum* (aqueous extract, 100 mg/mL, and leaf press juice) inhibited spontaneous contractions of human myometrial strips [19, 20]. In human myometrium cells, BPJ lowered the oxytocin-induced increase of intracellular calcium concentrations [21].

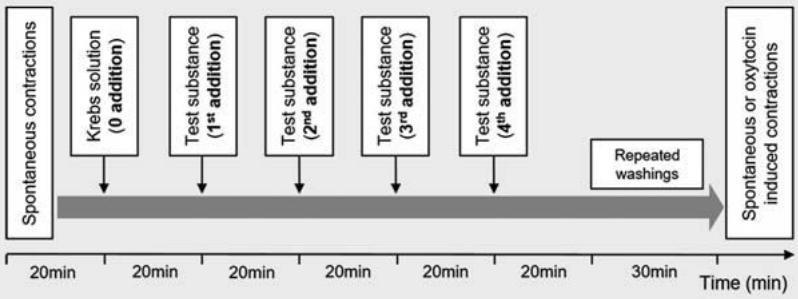
Flavonoid glycosides and bufadienolides are the major classes of secondary metabolites in *B. pinnatum* leaves [22, 23]. The presence of nine different glycosides of kaempferol, quercetin, myricetin, acacetin, and diosmetin was shown, and four bufadienolides, namely, bersaldegenin-1-acetate, bryophyllin A, bersaldegenin-3-acetate, and bersaldegenin-1,3,5-orthoacetate, were identified [22]. Fractions enriched in flavonoid glycosides (FEF) and bufadienolides (BEF) were prepared, and their effects on human myometrial contractility were characterised *in vitro*. The activity of fractions was compared with that of BPJ, the starting material for the *B. pinnatum* tablets used in clinical practice, and with a mixture of flavonoid aglycons (A-mix) that corresponded to the composition of aglycons in FEF.

Results

To investigate the contribution of different constituents of *B. pinnatum* leaves on human myometrial contractility, spontaneously contracting strips of myometrium mounted in an organ bath system were repeatedly exposed to increasing concentrations of FEF, A-Mix, BEF, and BPJ. A schematic representation of the experimental design is given in ▶ **Fig. 1**. The concentration ranges for FEF, BEF, and BPJ (▶ **Table 1**) were determined in preliminary experiments to ensure that an effect on the strength of contractions, measured as the AUC and amplitude, would be visible upon two to three additions. The concentration range of A-Mix was such that the concentration of the main flavonoid aglycons in the organ bath were identical to those of the corresponding flavonoid glycosides in FEF.

The AUC of myometrial contractions decreased progressively and in a statistically significant way with the repeated addition of FEF and the corresponding A-Mix (FEF: $p < 0.001$, A-Mix: $p < 0.001$; Friedman test) (▶ **Fig. 2A**). Compared to addition 0, the values of the AUC were significantly lower after the 3rd and 4th additions of FEF (final concentrations of 150 and 200 µg/mL, $p = 0.01$ and $p < 0.001$, respectively; Dunn's multiple comparisons test) (▶ **Fig. 2A**). The repeated addition of A-Mix also significantly lowered the AUC after the 3rd and 4th additions (concentrations of 6.2 and 8.3 µg/mL), again when compared to addition 0 ($p = 0.008$ and $p = 0.002$, respectively) (▶ **Fig. 2A**). Comparable inhibitory effects of FEF and A-Mix on the amplitude were observed (FEF: $p = 0.01$, A-Mix: $p < 0.001$) (▶ **Fig. 2B**). Compared to addition 0, the 4th addition of FEF and the 3rd addition of A-Mix led to significantly lower amplitudes (in each case $p = 0.01$) (▶ **Fig. 2B**). The repeated addition of FEF promoted a progressive increase in contraction frequency ($p = 0.001$), with significant differences after the 3rd and 4th additions ($p = 0.004$ and $p = 0.002$, respectively). In contrast, increased concentrations of A-Mix did not affect contraction frequency (▶ **Fig. 2C**).

As shown in ▶ **Fig. 3**, the effect of BEF on the AUC of myometrial contractility was concentration dependent ($p < 0.001$). When each addition was compared to addition 0, a significant difference was obtained with the 3rd and 4th additions (concentrations of 1.0 and 1.3 µg/mL, $p = 0.02$ and $p < 0.001$, respectively) (▶ **Fig. 3A**).



▶ **Fig. 1** Experimental design for measurement of myometrial contractions. When uterine strips were contracting regularly for 20 min, Krebs solution was added to the organ bath (addition 0) and contractility was recorded for 20 min. Thereafter, a test substance was added four times, at intervals of 20 min each, and contractility was recorded for 20 min after each addition. A washout period of 30 min followed, with a change of Krebs solution at 5, 10, 20, and 30 min. If the strip was not contracting spontaneously at the end of the washout period, oxytocin was added (final contraction 1 U/L).

▶ **Table 1** Side-by-side comparison of flavonoid aglycon and bufadienolide concentrations in the organ bath upon consecutive addition of the various test substances. Concentration of the flavonoid-enriched fraction (FEF), the corresponding flavonoid aglycon mixture (A-Mix), the bufadienolide-enriched fraction (BEF), and *B. pinnatum* leaf press juice (BPJ) in the organ bath after each addition is shown. The corresponding concentrations of flavonoid aglycons in FEF and BPJ, and bufadienolides in BEF and BPJ were calculated.

Addition	FEF		A-Mix	BEF		BPJ		
	(µg/mL)	Flavonoids (µg/mL)	(µg/mL)	(µg/mL)	Bufadienolides (µg/mL)	µg/mL	Flavonoids (µg/mL)	Bufadienolides (µg/mL)
1	50	2.07	2.07	0.33	0.030	3.3	0.11	0.033
2	100	4.14	4.14	0.67	0.061	6.7	0.23	0.067
3	150	6.21	6.21	1.00	0.091	10.0	0.34	0.100
4	200	8.28	8.28	1.33	0.121	13.3	0.45	0.133

The amplitude of the contractions also decreased with consecutive additions of BEF ($p = 0.001$) and was significantly different from addition 0 after the 4th addition (final concentration of BEF in the organ bath was 1.3 µg/mL, $p = 0.003$) (▶ **Fig. 3B**). The frequency of the myometrial contractions increased with the repeated additions of BEF ($p = 0.001$), with significant differences, relative to addition 0, being obtained after the 3rd and 4th additions ($p = 0.004$ and $p = 0.002$, respectively) (▶ **Fig. 3C**).

To compare the effects described above with the active ingredient of commercially available 50% *B. pinnatum* tablets, BPJ was also tested. The addition of BPJ led to a progressive decrease of the AUC compared to addition 0 ($p < 0.001$). After the 3rd and 4th additions [final concentrations of 10 and 13 µg/mL (1.0 and 1.3%) of BPJ], the values of the AUC were significantly lower than after addition 0 ($p = 0.01$ and $p < 0.001$, respectively) (▶ **Fig. 4A**). A progressive decrease of the amplitude was also observed ($p < 0.003$). As shown in ▶ **Fig. 4B**, the 4th addition of BPJ resulted in a significant decrease of contraction amplitudes compared to addition 0 ($p = 0.01$, final concentration of 13 µg/mL). BPJ led to a strong increase in contraction frequency ($p < 0.001$) to 860.0 ± 219.4% ($p = 0.007$) at the 3rd addition, and 1120.0 ± 214.2% of the initial value at the 4th addition (▶ **Fig. 4C**).

Nifedipine, a known tocolytic drug frequently used in clinical practice [7, 24], was used as the PC. The repeated addition of nifedipine led to a progressive decrease of the contraction strength assessed either as the AUC or as amplitude (in each case $p < 0.001$) (▶ **Fig. 4A, B**), with statistically significant values after the 3rd and 4th additions (concentrations of 16.2 and 21.6 nM) when compared to addition 0 (in each case $p = 0.01$ and $p = 0.0003$, respectively). The frequency of the myometrial contractions was not affected by increasing concentrations of nifedipine ($p < 0.5$) (▶ **Fig. 4C**).

The bufadienolide content in BEF and BPJ, and the flavonoid aglycons in FEF, had been previously determined [23, 25]. To be able to compare the effects of FEF with those of BPJ, the main flavonoid aglycons in BPJ were now determined by HPLC-PDA analysis after hydrolysis. BPJ contained 0.034 ± 0.006 mg/mL of flavonoid aglycons in the relative proportions of 78.7% quercetin, 4.8% myricetin, 11.5% diosmetin, and 4.9% kaempferol (**Fig. 1S**, Supporting Information).

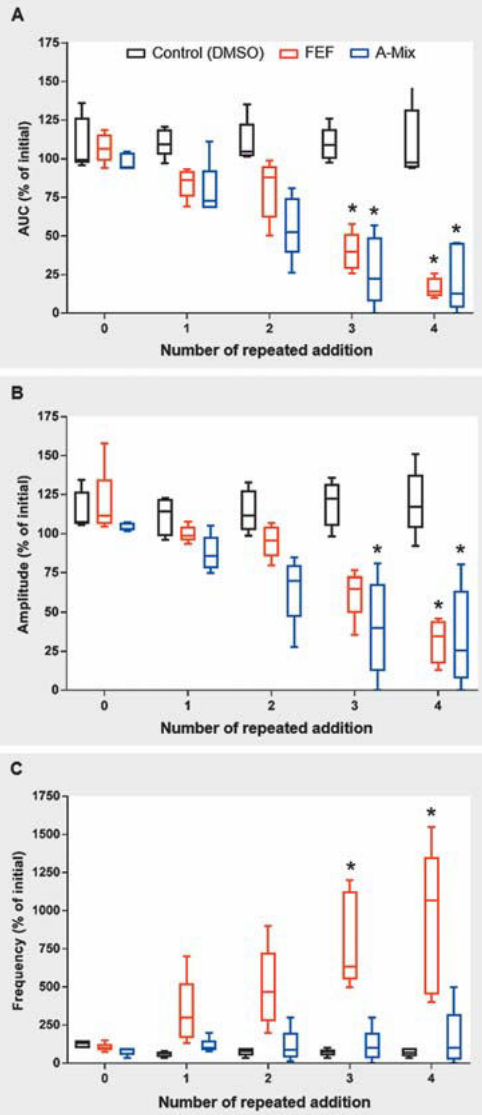
To verify whether the test substances could be toxic to myometrial tissue, the ability of the strips to contract again after the washing step at the end of the myograph experiments was determined. All strips contracted again (**Fig. 2S**, Supporting Information), either spontaneously or after the addition of oxytocin (final

concentration of 1 U/L). This indicated that test substances did not jeopardise the viability of the myometrium strips. In some myograph experiments, however, a decrease of contraction strength was still apparent after the washing step. Therefore, we assessed the cytotoxicity of the test substances during longer periods using two human myometrial cell lines. When hTERT myometrium cells were incubated with test substances for 24 h (data not shown) and 48 h, A-Mix, BEF, and BPJ did not affect cell viability at concentrations up to 40 µg/mL, 15 µg/mL, and 150 µg/mL, respectively. Moreover, FEF was cytotoxic only at a concentration of 1000 µg/mL when compared to the untreated control ($p < 0.05$) (▶ **Fig. 5**). When PHM1–41 cells were incubated with test substances for 24 h (data not shown) and 48 h, FEF and A-Mix did not affect cell viability at concentrations up to 1000 µg/mL and 40 µg/mL, respectively. BEF decreased cell viability when compared to the untreated control only after 48 h, at concentrations of 7.5 and 15 µg/mL ($p < 0.05$), while BPJ was cytotoxic only at 150 µg/mL ($p < 0.05$) (▶ **Fig. 5**). DMSO used to dissolve the test samples only decreased cell viability at concentrations higher than 1.5 % (**Fig. 3S**, Supporting Information).

Discussion

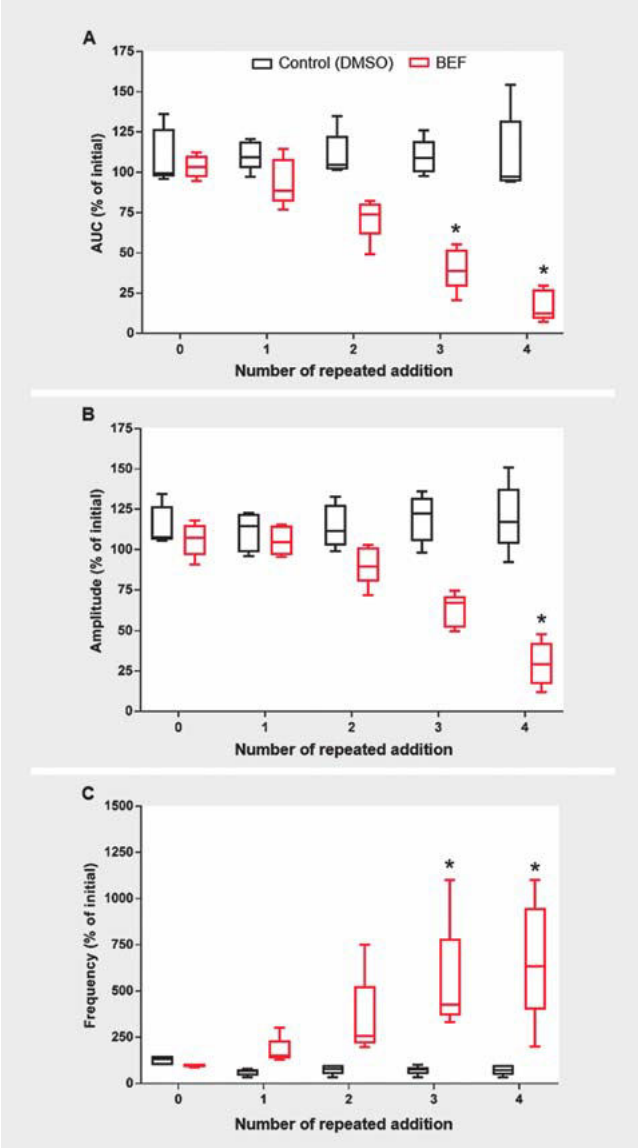
Fractions prepared from *B. pinnatum* leaves and enriched in flavonoid glycosides or in bufadienolides led to a concentration-dependent decrease of myometrial contraction strength *in vitro*. Therefore, our data suggest that FEF and BEF contain compounds that contribute to the inhibitory effect of BJP (▶ **Fig. 6**), the starting material for the 50 % *B. pinnatum* tablets that have been used in several prospective clinical studies [6, 8, 11, 12]. These *B. pinnatum* press juice tablets are taken orally, and the intestinal metabolisation of compounds thus needs to be considered. Flavonoid glycosides are known to be hydrolysed, leading to the release of flavonoid aglycons [26, 27], whereas bufadienolides in *B. pinnatum* are less likely to be metabolised by gut microbiota (own unpublished preliminary observations with bufalin). From a translational point of view, the similar abilities of FEF and A-mix to lower myometrium contractions *in vitro* suggest that the intestinal metabolisation step is not required for FEF effects on this tissue.

The concentrations in the organ bath of flavonoid aglycons after the addition of FEF and BPJ, and of bufadienolides after the addition of BEF and BPJ was calculated based on previous [23, 25] and present data, and are shown in ▶ **Table 1**. It is apparent that the concentration of flavonoid aglycons in BPJ additions was markedly lower than with FEF. In contrast, bufadienolide concentrations after the addition of BEF and BPJ were comparable. The repeated addition of FEF, A-Mix, BEF, and BPJ led to a comparable lowering of the AUC and amplitude (▶ **Fig. 6**), albeit at much higher concentrations for FEF and A-mix. From a translational point of view, this suggests that bufadienolides are mainly responsible for the inhibitory effect of BPJ on myometrium contractility, and that flavonoids only play a minor role. Therefore, special attention should be paid to the amount of bufadienolides present in *B. pinnatum* preparations administered for the prevention of preterm birth. In general, bufadienolides increasingly appear to be the major pharmacologically active compounds in *Bryophyllum* spp. [28].

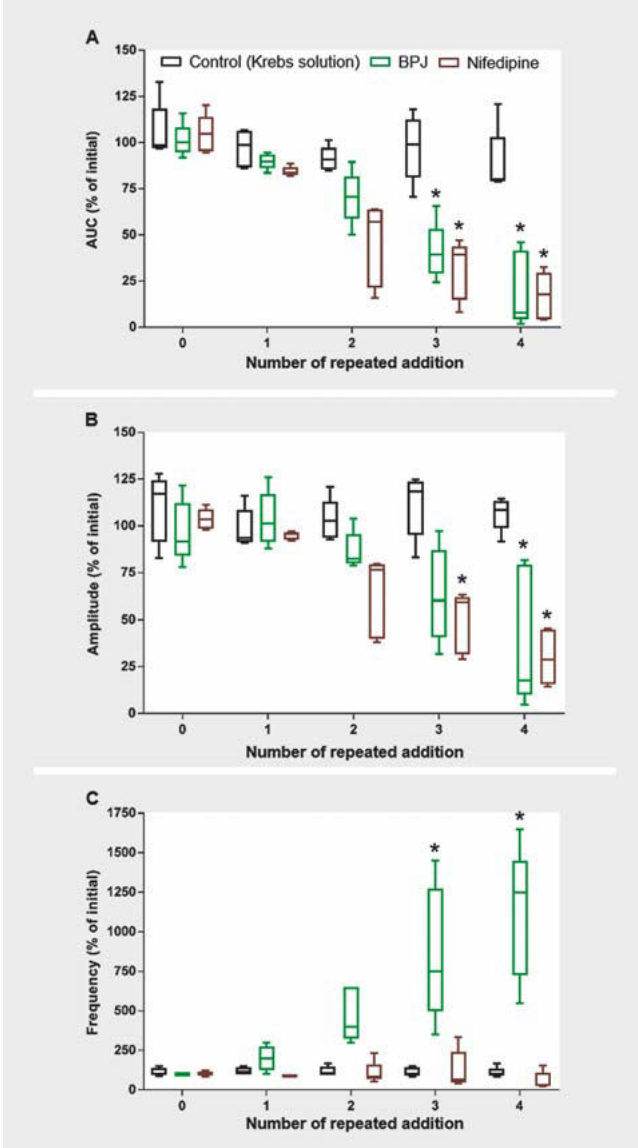


▶ **Fig. 2** Effect of repeated addition of the flavonoid-enriched fraction (FEF) and corresponding flavonoid aglycon mixture (A-Mix) on human myometrial contractility *in vitro*. FEF (2 µL of 150 mg/mL DMSO stock solution) or the corresponding A-Mix (2 µL of 6.21 mg/mL DMSO stock solution) was repeatedly added to the myograph chamber. The box plot shows the AUC (**A**), amplitude (**B**), and frequency (**C**). The bottom and top edges of the box are the 25th and 75th percentile, respectively, the central mark indicates the median, and the end of the whiskers represents minimum and maximum values. Data were obtained with five different biopsies (n = 5) and are expressed as percentage of the initial value. The repeated addition of DMSO (2 µL) was used as a control; *$p < 0.05$.

Human myometrium strips are the most relevant model for assessing the effects of substances on uterine contractions. The downside of the model is the highly limited availability of myometrium strips (they have to be freshly taken from a Caesarean section, with prior consent) and the very low throughput that can be achieved with an organ bath model. To study concentration dependency in this model, we have therefore increased the concen-



▶ **Fig. 3** Effect of repeated addition of the bufadienolide-enriched fraction (BEF) on human myometrial contractility *in vitro*. BEF (2 µL of 1 mg/mL DMSO stock solution) was repeatedly added to the myograph chamber. The box plot shows the AUC (**A**), amplitude (**B**), and frequency (**C**). Data were obtained with five different biopsies (n = 5) and are expressed as percentage of the initial value. The repeated addition of DMSO (2 µL) was used as acontrol; *$p < 0.05$.

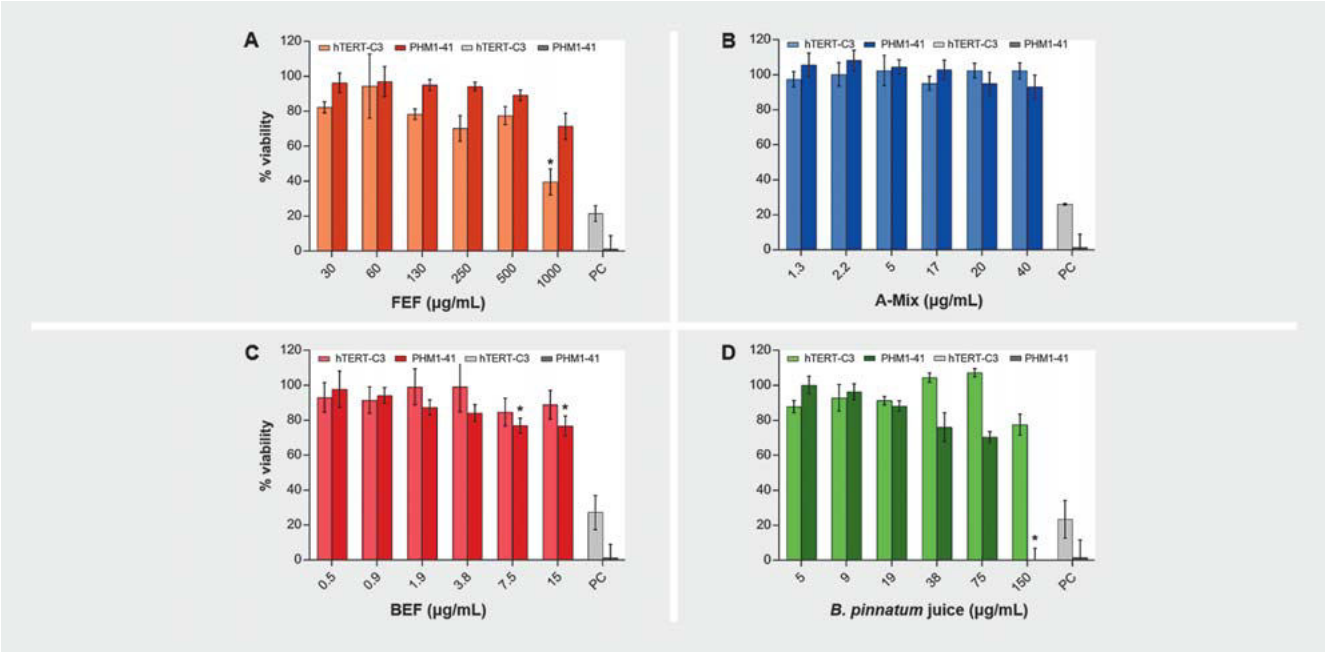


▶ **Fig. 4** Effect of repeated addition of *B. pinnatum* leaf press juice (BPJ) and nifedipine (positive control; PC) on human myometrial contractility *in vitro*. BPJ (20 µL) or nifedipine (3 µL of 10.8 µM) were repeatedly added to the myograph chamber. The box plot shows the AUC (**A**), amplitude (**B**), and frequency (**C**). Data were obtained with five different biopsies (n = 5) and are expressed as percentage of the initial value. The repeated addition of Krebs solution (20 µL) was used as a control; *$p < 0.05$.

tration of the various test substances in the myograph chamber with consecutive additions. The inclusion of vehicle (and concomitantly time) controls in the various experiments supported the concentration dependency of the effects. Moreover, the intervals of 20 min after each addition allow the compounds to penetrate into the tissue and thus to exert their effect.

The results obtained in the organ bath seem to represent a true inhibition of contractions since myometrium strips were still vital after the washout, i.e., they could still contract if given enough time and/or if stimulated with oxytocin (**Fig. 2S**, Supporting Information). Since the contraction strength of the strip treatment with the test substances was still lower than prior to the additions, we investigated the cytotoxic effects of the test substances on two human myometrium cell lines (hTERT-C3 and PHM1–41). The test substances were not cytotoxic even at concentrations far higher than those used in the organ bath experiments, and exposure times that were significantly longer (▶ **Figs. 5** and **3S**, Supporting Information).

Our results with the myometrium model show that the fractions obtained from *B. pinnatum* leaves (FEF and BEF) not only re-



▶ **Fig. 5** Cytotoxic effect of the flavonoid-enriched fraction (FEF, 30–1000 µg/mL) (**A**), the corresponding flavonoid aglycon mixture (A-Mix, 1.3–40 µg/mL) (**B**), the bufadienolide-enriched fraction (BEF, 0.5–15 mg/mL) (**C**), or *B. pinnatum* leaf press juice (BPJ, 0.5–15.0 %) (**D**) on human myometrium cell lines (hTERT-C3 and PHM1–41). Viability assays were performed after 24 and 48 h, and ethyl methanesulfonate was used as a positive control (PC). Data is presented as the mean ± SEM of four independent experiments carried out at least in quadruplicate (n = 4); $^{*}p < 0.05$.

duced contraction strength, but also increased the frequency of spontaneous contractions (▶ **Fig. 6**). This is in line with previous studies [19, 20], but different from what was observed with A-mix. The reason for the frequency increase with BPJ, FEF, and BEF is not clear at this point in time. As for the clinical practice, there are no published reports that *B. pinnatum* preparations would lead to an increase in frequency of the strong and rhythmical contractions that eventually lead to delivery.

Taken together, our data corroborate the relaxant properties of BPJ and fractions on myometrium strips and, therefore, support the use of *B. pinnatum* preparations in the management of preterm labour. Considering the qualitative and quantitative composition of the press juice and the fractions tested on one side, and the potency of relaxant effects on the other, bufadienolides appear to be the major active compounds of *B. pinnatum*.

Material and Methods

Chemicals

Quercetin (purity ≥ 95 %), myricetin (purity ≥ 96 %), diosmetin (purity ≥ 98 %), kaempferol (purity ≥ 97 %), $NaHCO_3$, glucose (ACS grade), KCl (purity ≥ 99 %), and EDTA were obtained from Sigma-Aldrich. KH_2PO_4 (purity ≥ 99.5 %) and $CaCl_2$ (purity ≥ 95 %) were purchased from Merck. $MgSO_4$ (purity ≥ 99 %) was purchased from Fluka and NaCl (purity ≥ 99.5 %) from PanReac AppliChem.

Plant material

Plant material originated from two different harvests. Weleda Brazil provided leaves harvested on 25 March 2014 in S. Roque, Brazil. Identification of this material was done by Moacyr Copani and Paulo Copani, Weleda Brazil. A voucher specimen (ZSS 29717) has been deposited at the Zurich Succulent Plant collection. Leaves were sent by airmail to Weleda AG, Arlesheim, Switzerland, in a refrigerated box. In addition, the Weleda branch located in Schwäbisch Gmünd, Germany, provided leaves harvested in July and August 2010. Identification of this material was done by Michael Straub, Weleda Germany. A voucher specimen (ZSS 29715) was deposited at the Zurich Succulent Plant collection. After harvesting, the leaves were frozen and stored at − 20 °C until processing.

Bryophyllum pinnatum leaf press juice

BPJ was prepared from leaves harvested in S. Roque, Brazil. The press juice was obtained by the mechanical pressing of the 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 % chewing tablets (Weleda AG). Unfiltered press juice was kept at − 80 °C until use.

Flavonoid- and bufadienolide-enriched fractions

FEF and BEF used in the present study originated from earlier investigations, and chromatograms of these fractions have been published (see [29] and [25], respectively). Briefly, frozen fresh leaves of *B. pinnatum* (from Schwäbisch Gmünd, Germany) were lyophilised and powdered in a mortar, and the powder (1065 g) was extracted with MeOH. After evaporation, a portion of the MeOH extract (112 g) was partitioned between CH_2Cl_2 and H_2O, and the aqueous phase (2.2 g) was fractionated by column chromatography on Diaion HP-20 to provide the FEF (610 mg). Evapo-

ration of the CH_2Cl_2 phase yielded a residue (10.8 g) [29] that was purified by solid-phase extraction on RP-18 to afford 268 mg of BEF [23]. For testing, FEF and BEF stock solutions (150 mg/mL and 1.0 mg/mL, respectively) were prepared in DMSO.

Flavonoid aglycon mix

A content of 4.14% of total flavonoid aglycons after hydrolysis of FEF was previously determined, and the relative proportions were 74.6% quercetin, 16.7% myricetin, 4.6% diosmetin, and 4.0% kaempferol [25]. A mixture of the four aglycons in these proportions (A-Mix) was prepared in DMSO at a concentration of 6.21 mg/mL.

Acid hydrolysis of *Bryophyllum pinnatum* leaf press juice and quantification of flavonoid aglycons

Concentrated HCl (4.05 mL) was added to *B. pinnatum* juice (20 mL) to a final concentration of 2 N. The mixture was heated under reflux at 90 °C for 1.5 h. The reaction mixture was allowed to cool and was neutralised with $NaHCO_3$. The solution was extracted with ethyl acetate (3 × 20 mL), and the combined organic layers were evaporated under reduced pressure. The solid residue was redissolved in DMSO (5 mL). Hydrolysis was performed in triplicate.

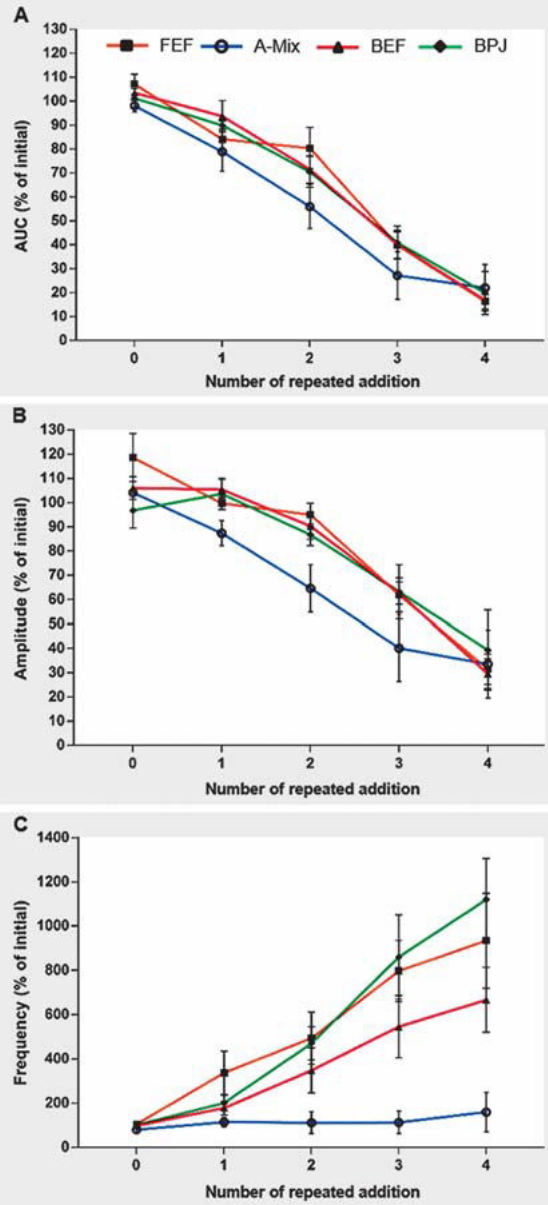
HPLC analysis was performed on a Shimadzu LCMS 8030 system with a photodiode array detector using an Atlantis dC18 column (4.6 × 150 mm, 3 µm; Waters). The mobile phase consisted of H_2O (A) and MeOH (B), both containing 0.1% formic acid. After isocratic elution with 50% B for 2 min, a gradient of 50–75% B in 28 min was applied. The flow rate was 0.6 mL/min, and detection was at 254 nm. The injection volume was 10 µL. The identity of the aglycons was confirmed by electrospray ionisation mass spectrometry and by comparison with reference standards. Quantification of the five aglycons was performed using a calibration curve for quercetin (12.5–200 µg/mL) and a previously determined response factor [25]. The HPLC-UV chromatogram of the hydrolysed juice and the calibration curve are shown as Supporting Information (**Fig. 1S**, Supporting Information).

Measurement of myometrial contractility *in vitro*

The study was approved by the ethics committee of canton Zurich (KEK-ZH-Nr. 2014–0717). Written informed consent of the study participants was obtained on the day before surgery. Inclusion criteria were planned first caesarean section, single pregnancy, negative HIV test, age > 18 years, and no tocolysis within 2 weeks before the caesarean section.

A myometrial biopsy of approximately 5 g was taken from each study participant at the cranial edge of the uterotomy during the elective caesarean section. The myometrial biopsies were immediately stored in Ringer solution and transferred to the laboratory for experiments.

Longitudinal muscle strips of approximately 15 × 2 × 1 mm were prepared and mounted between two clamps of a myograph bath chamber containing 6 mL of Krebs solution (118 mM NaCl, 24.9 mM $NaHCO_3$, 4.7 mM KCl, 1.24 mM KH_2PO_4, 2.48 mM $CaCl_2$, 1.21 mM $MgSO_4$, 10 mM glucose, 0.034 mM EDTA, pH = 7.4), with the temperature set at 37 °C, and aerated with 95% O_2 and 5% CO_2 (PanGas). Contractions were recorded by a DMT800MS myograph (Muscle Strip Myograph system, DMT, ADInstruments) and transferred to a PC via a transducer (ADInstruments PowerLab 4/30).



▶ **Fig. 6** Comparison of the effects of the flavonoid-enriched fraction (FEF), the corresponding flavonoid aglycon mixture (A-Mix), the bufadienolide-enriched fraction (BEF), and *B. pinnatum* leaf press juice (BPJ) on human myometrial contractility *in vitro*. For experimental details, see legends to ▶ **Figs. 2**, **3**, and **4**.

Regular spontaneous contractions were recorded for 20 min. Then, Krebs solution was added (addition 0), and contractility was recorded for 20 min. Each strip was treated with 1 test substance by adding 4 times, at time intervals of 20 min, the same volume of a stock solution of the test compound, and recording contractility for 20 min after each addition. Test solutions included: control, 20 µL Krebs solution or 2 µL DMSO; FEF, 2 µL of

150.0 mg/mL; A-Mix, 2 µL of 6.21 mg/mL; BEF, 2 µL of 1.0 mg/mL; or BPJ, 20 µL. Contractions as a measure of vitality of the tissue was determined after a 30-min period of washing, where Krebs solution was changed at 5, 10, 20, and 30 min (▶ **Fig. 1**). If the strip did not contract spontaneously after the washing, 1 U of oxytocin was added to stimulate contractions. After observing contractility, the experiment was stopped. Nifedipine was used as a positive control (3 µL of 10.8 µM in Krebs solution). For each substance tested, five different biopsies were used (n = 5).

Myograph data processing

Myometrium contractions were recorded by LabChart Pro 8.0.6 (ADInstruments) and analysed with the peak analysis module. AUC and amplitude of each contraction were calculated. Average AUC and average amplitude were taken as measures for the strength of contractility in each of the 20-min phases. The number of contractions in each phase was also recorded (frequency). Spontaneous contractions (before any addition, i.e., baseline) were set at 100%. The effect after addition was expressed as percentage of the initial value. The values of the various biopsies were used for further statistical analysis (n = 5 per test substance).

Cell culture

A human myometrial telomerase reverse transcriptase cell line (hTERT-C3) [30, 31], provided by M. Grãos (University of Coimbra, Portugal), was cultured in a 1 : 1 mixture of DMEM and F-12 supplemented with antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and 10% (v/v) heat-inactivated FBS (all from Gibco). Human uterine myometrium smooth muscle cells (PHM1-41), obtained from ATCC (CRL-3046) were maintained in ATCC-formulated DMEM (ATCC No. 30-2002) supplemented with 0.1 mg/mL G-418 (Carl Roth), 2 mM glutamine, and 10% (v/v) heat-inactivated FBS (Gibco).

Cell viability

Cells were seeded into transparent 96-well microplates. hTERT-C3 cells were seeded at a density of 5×10^4 cells/mL (5×10^3 cells per well) and PHM1–41 at a density of 8×10^4 cells/mL (8×10^3 cells per well). One day after seeding, cells were exposed to FEF (30–1000 µg/mL), A-Mix (1.3–40 µg/mL), BEF (0.5–15 µg/mL), BPJ (5–150 µg/mL), and DMSO (0.2–6.0%) for 24 and 48 h. After exposure, resazurin was added to the cells (final concentration 1.0 mg/mL), and the plate was incubated at 37 °C for 4 h. The extent of resazurin reduction was measured in a microplate reader (SpectraMax Paradigm, Molecular Devices) at 570 and 600 nm. For each substance tested, four independent experiments were carried out at least in quadruplicate. Ethyl methanesulfonate (30 mM) [32] was used as a positive control. In each experiment, wells with no test substance added to the culture medium served as an untreated control (100% viability). Cell viability was determined according to the following equation:

$$\text{Viability} = \frac{(A_{570} - A_{600})\text{sample} - (A_{570} - A_{600})\text{blank}}{(A_{570} - A_{600})\text{control} - (A_{570} - A_{600})\text{blank}}$$

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. For each test substance, myograph measurements (n = 5) and cell viability data (n = 4) were analysed with the paired, non-parametric Friedman test. In all cases, this test was followed by Dunn's multiple comparisons test in which the various additions or concentrations were compared to addition 0 or untreated control, respectively. A significance level of $p < 0.05$ was considered statistically significant. Myograph measurements data are presented as box plots (median, 25th percentile and 75th percentile, and minimum and maximum values) and as the mean ± standard error of the mean (SEM). Cell viability data is given as the mean ± SEM.

Supporting information

An HPLC-UV chromatogram of hydrolysed BPJ, a calibration curve for quercetin, and the effect of different concentrations of DMSO on the human myometrium hTERT-C3 and cell line PHM1-41 are available as Supporting Information.

Acknowledgements

The authors are grateful to Alexandra Dolder for technical support, and to the staff at the Department of Obstetrics, University Hospital Zurich, who facilitated the collection of myometrium samples. We are indebted to the members of the Bryophyllum Study Group for critical discussions, and to Karin Fürer for preparation of FEF and of a bufadienolide-enriched fraction that was eventually purified to obtain BEF. Financial support was provided by Weleda AG and the Johannes Kreyenbühl Foundation.

Conflict of Interest

M. M. is an employee of Weleda AG, the company that produces preparations of *B. pinnatum*. A. P. S. W. received research funding from Weleda AG during the last 5 years.

References

[1] Kamboj A, Rathour A, Kaur M. Bufadienolides and their medicinal utility: a review. Int J Pharm Pharm Sci 2013; 5: 20–27

[2] Biswas SK, Chowdhury A, Das J, Hosen SZ, Uddin R, Rahaman MS. Literature review on pharmacological potentials of *Kalanchoe pinnata* (Crassulaceae). Afr J Pharm Pharmacol 2011; 5: 1258–1262

[3] Hamburger M, Potterat O, Fürer K, Simões-Wüst AP, von Mandach U. *Bryophyllum pinnatum*-reverse engineering of an anthroposophic herbal medicine. Nat Prod Commun 2017; 12: 1359–1364

[4] Fürer K, Simões-Wüst AP, von Mandach U, Hamburger M, Potterat O. *Bryophyllum pinnatum* and related species used in anthroposophic medicine: constituents, pharmacological activities, and clinical efficacy. Planta Med 2016; 82: 930–941

[5] Hassauer W, Schreiber K, von der Decken D. Ein neuer Weg in der tokolytischen Therapie. Erfahrungsheilkunde 1985; 34: 683–687

[6] Fürer K, Simões-Wüst AP, Winkler A, Amsler N, Schnelle M, von Mandach U. Die Anwendung von *Bryophyllum pinnatum*-Präparaten in der Geburtshilfe und Gynäkologie – eine multizentrische prospektive Beobachtungsstudie. Complement Med Res 2015; 22: 231–236

[7] Schenkel L, Simões-Wüst AP, Hösli I, von Mandach U. Drugs in pregnancy and lactation – medications used in Swiss obstetrics. Z Geburtshilfe Neonatol 2018; 222: 1–14

[8] Betschart C, von Mandach U, Seifert B, Scheiner D, Perucchini D, Fink D, Geissbühler V. Randomized, double-blind placebo-controlled trial with *Bryophyllum pinnatum* versus placebo for the treatment of overactive bladder in postmenopausal women. Phytomedicine 2013; 20: 351–358

[9] Lambrigger-Steiner C, Simões-Wüst AP, Kuck A, Fürer K, Hamburger M, von Mandach U. Sleep quality in pregnancy during treatment with *Bryophyllum pinnatum*: an observational study. Phytomedicine 2014; 21: 753–757

[10] Plangger N, Rist L, Zimmermann R, von Mandach U. Intravenous tocolysis with *Bryophyllum pinnatum* is better tolerated than beta-agonist application. Eur J Obstet Gynecol Reprod Biol 2006; 124: 168–172

[11] Simões-Wüst AP, Lapaire O, Hösli I, Wächter R, Fürer K, Schnelle M, Mennet-von Eiff M, Seifert B, von Mandach U. Two randomised clinical trials on the use of *Bryophyllum pinnatum* in pre-term labour – results after discontinuation. Complement Med Res 2018; 25: 269–273

[12] Simões-Wüst AP, Al Hassani T, Müller-Hübenthal B, Pittl S, Kuck A, Meden H, Eberhard J, Decker M, Fürer K, von Mandach U. Sleep quality improves during treatment with *Bryophyllum pinnatum*: an observational study on cancer patients. Integr Cancer Ther 2015; 14: 452–459

[13] Chang HH, Larson J, Blencowe H, Spong CY, Howson CP, Cairns-Smith S, Lackritz EM, Lee SK, Mason E, Serazin AC. Preventing preterm births: trends and potential reductions with current interventionsin 39 very high human development index countries. Lancet 2013; 381: 223

[14] World Health Organization. Preterm birth. Available at http://www.who.int/mediacentre/factsheets/fs363/en/. Accessed April 11, 2018

[15] Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes. Science 2014; 345: 760–765

[16] Schleußner E. The prevention, diagnosis and treatment of premature labor. Dtsch Ärztebl Int 2013; 110: 227

[17] Hubinont C, Debiève F. Prevention of preterm labour: 2011 update on tocolysis. J Pregnancy 2011; 2011: 941057

[18] ACOG: American college of Obstetricians and Gynecologists. Practice Bulletin No. 171: Management of preterm Labor. Obstet Gynecol 2016; 128: e155–e164

[19] Gwehenberger B, Rist L, Huch R, von Mandach U. Effect of *Bryophyllum pinnatum* versus fenoterol on uterine contractility. Eur J Obstet Gynecol Reprod Biol 2004; 113: 164–171

[20] Wächter R, Brenneisen R, Hamburger M, Mennet M, Schnelle M, Worel A, Simões-Wüst AP, von Mandach U. Leaf press juice from *Bryophyllum pinnatum* (Lamarck) Oken induces myometrial relaxation. Phytomedicine 2011; 19: 74–82

[21] Simões-Wüst AP, Grãos M, Duarte C, Brenneisen R, Hamburger M, Mennet M, Ramos M, Schnelle M, Wächter R, Worel A. Juice of *Bryophyllum pinnatum* (Lam.) inhibits oxytocin-induced increase of the intracellular calcium concentration in human myometrial cells. Phytomedicine 2010; 17: 980–986

[22] Fürer K, Raith M, Brenneisen R, Mennet M, Simões-Wüst AP, von Mandach U, Hamburger M, Potterat O. Two new flavonol glycosides and a metabolite profile of *Bryophyllum pinnatum*, a phytotherapeutic used in obstetrics and gynaecology. Planta Med 2013; 79: 1565–1571

[23] Oufir M, Seiler C, Gerodetti M, Gerber J, Fürer K, Mennet-von Eiff M, Elsas SM, Brenneisen R, von Mandach U, Hamburger M. Quantification of bufadienolides in *Bryophyllum pinnatum* leaves and manufactured products by UHPLC-ESIMS/MS. Planta Med 2015; 81: 1190–1197

[24] Vogel JP, Nardin JM, Dowswell T, West HM, Oladapo OT. Combination of tocolytic agents for inhibiting preterm labour. Cochrane Database Syst Rev 2014; (7): CD006169

[25] Bachmann S, Betschart C, Gerber J, Fürer K, Mennet M, Hamburger M, Potterat O, von Mandach U, Simões-Wüst AP. Potential of *Bryophyllum pinnatum* as a detrusor relaxant: an *in vitro* exploratory study. Planta Med 2017; 83: 1274–1280

[26] Cassidy A, Minihane AM. The role of metabolism (and the microbiome) in defining the clinical efficacy of dietary flavonoids. Am J Clin Nutr 2017; 105: 10–22

[27] Braune A, Blaut M. Bacterial species involved in the conversion of dietary flavonoids in the human gut. Gut Microbes 2016; 7: 216–234

[28] Kolodziejczyk-Czepas J, Stochmal A. Bufadienolides of *Kalanchoe* species: an overview of chemical structure, biological activity and prospects for pharmacological use. Phytochem Rev 2017; 16: 1155–1171

[29] 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. Inhibition of porcine detrusor contractility by the flavonoid fraction of *Bryophyllum pinnatum* – a potential phytotherapeutic drug for the treatment of the overactive bladder syndrome. Phytomedicine 2015; 22: 158–164

[30] Condon J, Yin S, Mayhew B, Word RA, Wright W, Shay J, Rainey WE. Telomerase immortalization of human myometrial cells. Biol Reprod 2002; 67: 506–514

[31] Devost D, Zingg HH. Novel *in vitro* system for functional assessment of oxytocin action. Am J Physiol Endocrinol Metab 2007; 292: E1–E6

[32] Ray SD, Fariss MW. Role of cellular energy status in tocopheryl hemisuccinate cytoprotection against ethyl methanesulfonate-induced toxicity. Arch Biochem Biophys 1994; 311: 180–190

Supporting Information

A Bufadienolide-Enriched Fraction of *Bryophyllum pinnatum* Inhibits Human Myometrial Contractility *In Vitro*

Stefanie Santos[1, 2], Christian Haslinger[1], Kristian Klaic[1], Maria T. Faleschini[2], Mónica Mennet[3], Olivier Potterat[2], Ursula von Mandach[1], Matthias Hamburger[2], Ana Paula Simões-Wüst[1]

Affiliations

[1]Department of Obstetrics, University Hospital Zurich, Zurich, Switzerland

[2]Division of Pharmaceutical Biology, University of Basel, Basel, Switzerland

[3]Weleda AG, Arlesheim, Switzerland

Correspondence

PD Dr. Ana Paula Simões-Wüst

University Hospital of Zurich

Department of Obstetrics

Schmelzbergstrasse 12/PF 125

Path G51a

8091 Zurich

Switzerland

Phone: +41 44 255 5131

Fax: +41 44 255 4430

anapaula.simoes-wuest@usz.ch

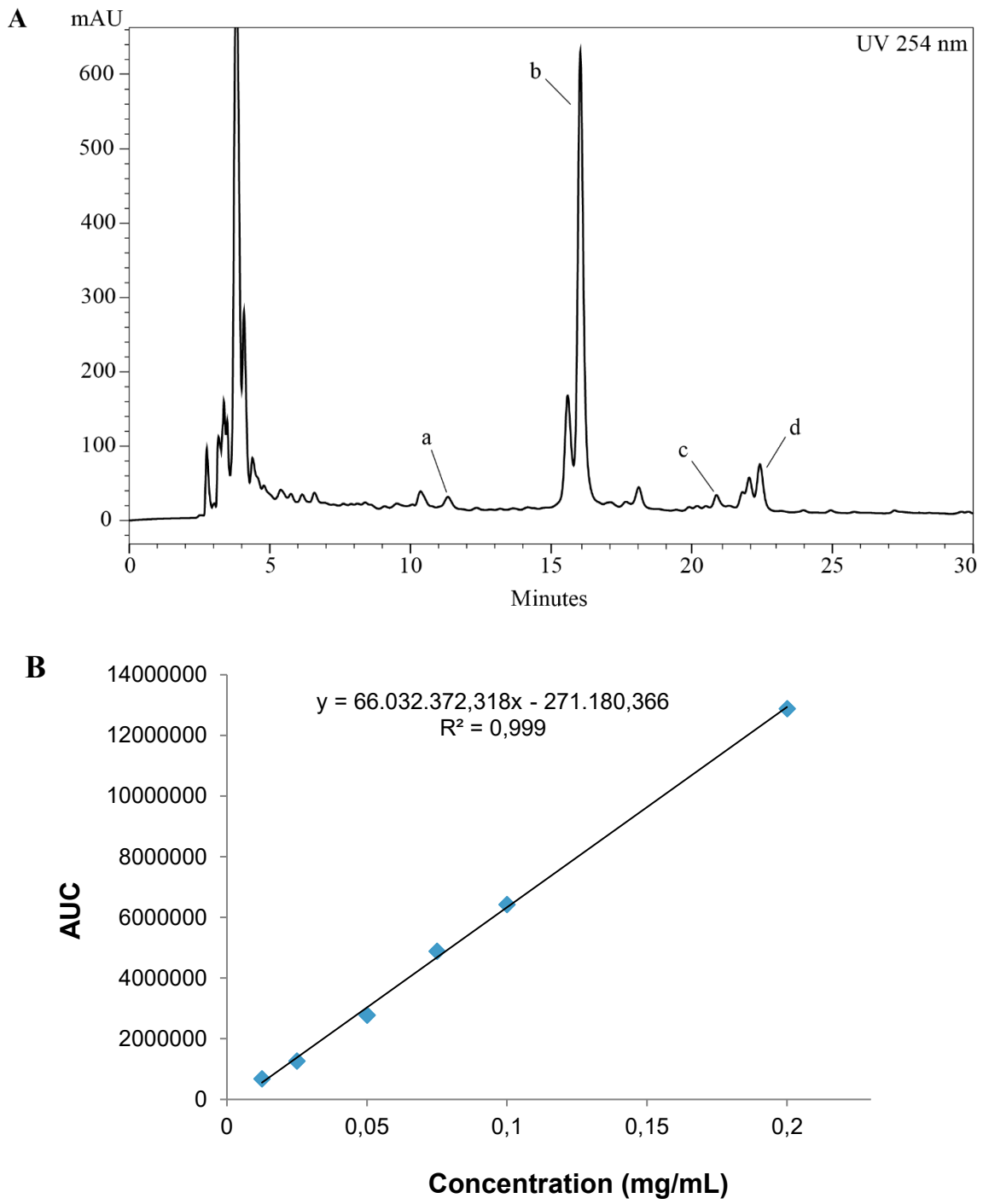


Fig. 1S A HPLC chromatogram of the hydrolysed juice. Myricetin (**a**), quercetin (**b**), kaempferol (**c**), diosmetin (**d**). Analyses were performed using an Atlantis dC-18 column (4.6 × 100 mm, 3 µm; Waters) with H_2O + 0.1% formic acid (A) and MeOH + 0.1% formic acid (B). Isocratic elution with 50% B for 2 min, then 50-75% B in 28 min; 0.6 mL/min. Detection was at UV 254 nm. **B** Calibration curve of quercetin (0.0125-0.2 µg/mL)

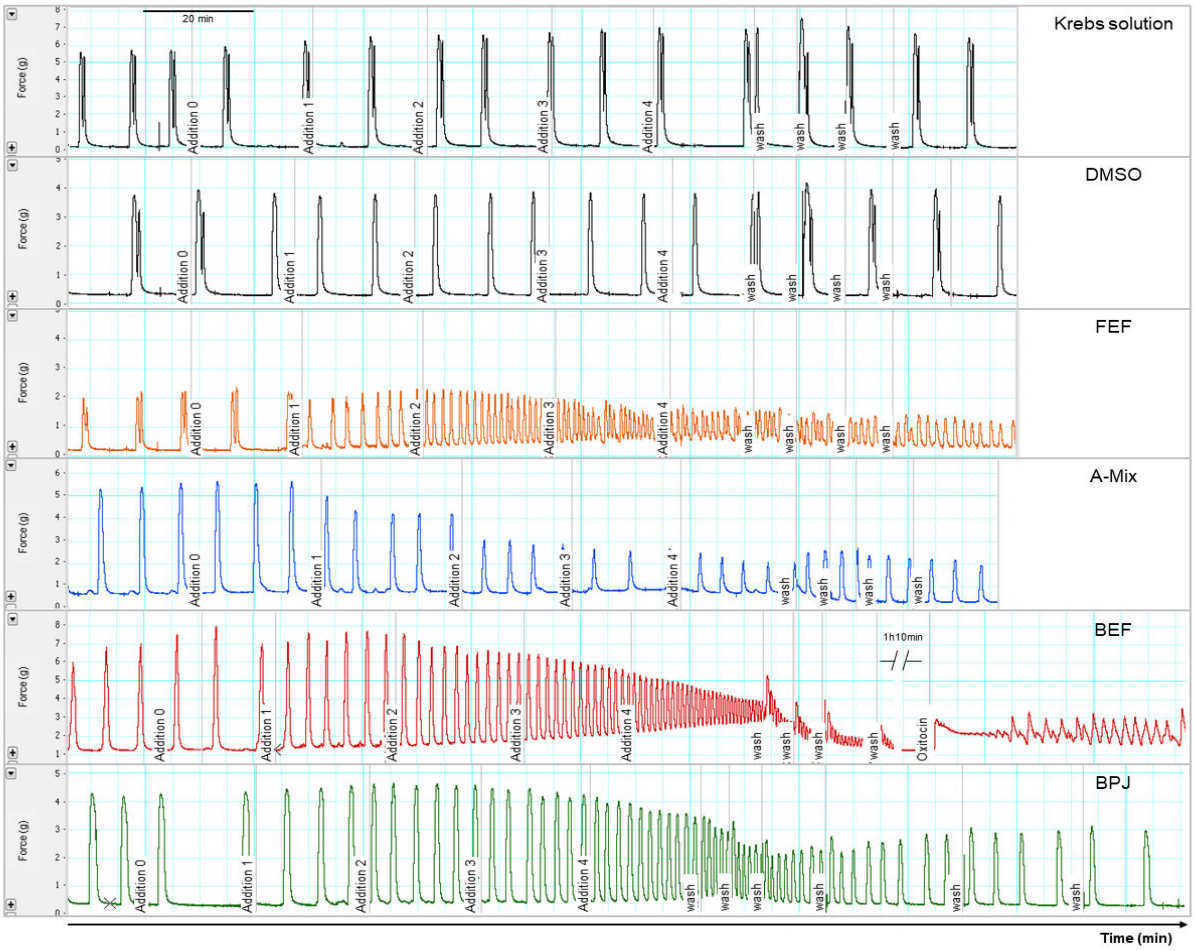


Fig. 2S Effect of repeated addition of the flavonoid-enriched fraction (FEF) and corresponding flavonoid aglycon mixture (A-Mix), the bufadienolide-enriched fraction (BEF), *B. pinnatum* leaf press juice (BPJ), and the corresponding controls (Krebs solution and DMSO) on human myometrial contractility *in vitro*. Krebs solution (20 µL), DMSO (2 µL), FEF (2 µL of 150 mg/mL DMSO stock solution), A-Mix (2 µL of 6.21 mg/mL DMSO stock solution), BEF (2 µL of 1 mg/mL DMSO stock solution), or BPJ (20 µL) were repeatedly added to the myograph chamber. When uterine strips were contracting regularly for 20 min, Krebs solution was added to the organ bath (addition 0) and contractility was recorded for 20 min. Thereafter, a test substance was added four times at intervals of 20 min each, and contractility was recorded for 20 min after each addition. A washout period of 30 min followed, with a change of Krebs solution at 5, 10, 20, and 30 min. If the strip was not contracting spontaneously at the end of the washout period, oxytocin was added (final contraction 1 U/L). Data are from one representative experiment out of five.

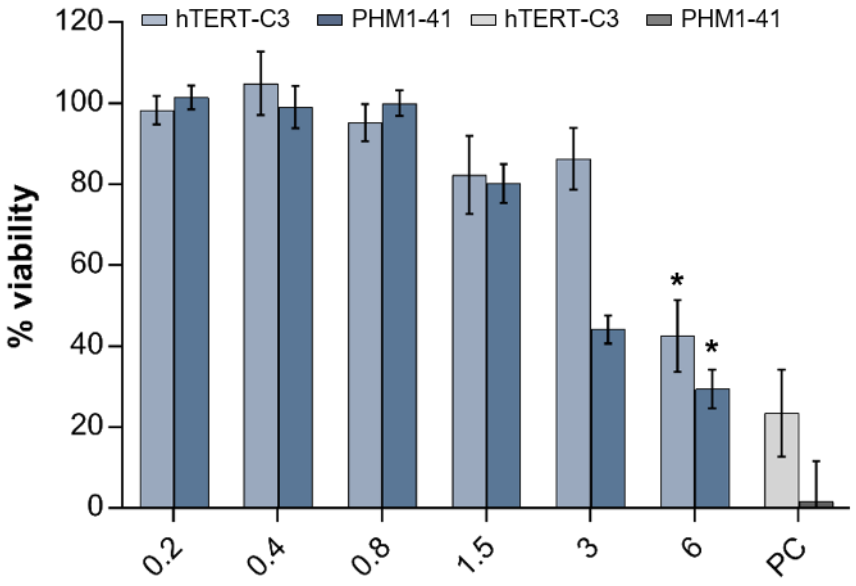


Fig. 3S Cytotoxic effects of DMSO (0.2-6.0%) on human myometrium cells (hTERT-C3 and PHM1-41). Viability assays were performed after a 48-h incubation. Ethyl methanesulfonate was used as a positive control (PC). Data are presented as the mean ± SEM of four independent experiments (n = 4) carried out at least in quadruplicate; *$p < 0.05$.

Chapter 3

Oxytocin induced pathways

Drawing from Clipart Library.

3.1. *Bryophyllum pinnatum* compounds inhibit oxytocin-induced signalling pathway in human myometrial cells

Stefanie Santos, Leonie Zurfluh, Mónica Mennet, Olivier Potterat, Ursula von Mandach, Matthias Hamburger, Ana Paula Simões-Wüst

Submitted to: *Scientific Reports*

Human myometrial cells (hTERT-C3) loaded with a calcium specific fluorescent probe (FURA-2 AM) were pre-incubated with a flavonoid-enriched fraction (FEF), the corresponding flavonoid aglycon mixture (A-Mix), a bufadienolide-enriched fraction (BEF), bersaldegenin-1,3,5-orthoacetate (BO), the combination of BEF plus FEF, the combination of BEF plus A-Mix, *B. pinnatum* leaf press juice (BPJ), and the oxytocin (OT)-receptor antagonist atosiban. The Combination Index (CI) test was used to characterise combined effects. To confirm that results were not cell specific, the effect of BPJ and atosiban were also studied in another cell line (PHM1-41). The phosphorylation of MAPKs p38, SAPK/JNK, and ERK1/2 was examined by Western blot. hTERT-C3 cells were incubated with BPJ, FEF, A-Mix, BEF, different combinations, and BPJ for 30 min and then stimulated with OT.

My contributions to this publication: cultivation of cells, design and performance of cell experiments, help master student perform cell experiments, recording and analysing data, writing the manuscript draft, and preparation of figures and tables.

Stefanie Lopes dos Santos

Bryophyllum pinnatum compounds inhibit oxytocin-induced signalling pathways in human myometrial cells

S. Santos[1,2], L. Zurfluh[1], Mónica Mennet[3], O. Potterat[2], U. von Mandach[2], M. Hamburger[2], A.P. Simões-Wüst[1*]

[1]Department of Obstetrics, University Hospital Zurich, Zurich, Switzerland

[2]Division of Pharmaceutical Biology, University of Basel, Basel, Switzerland

[3]Weleda AG, Arlesheim, Switzerland

*Corresponding author.

PD Dr. Ana Paula Simões-Wüst, University Hospital of Zurich, Department of Obstetrics, Schmelzbergstrasse 12/PF 125, Path G51a, 8091 Zurich, Switzerland; phone: +41 44 2555131; Fax: +41 44 2554430; anapaula.simoes-wuest@usz.ch

Abstract

Bryophyllum pinnatum has been used in the treatment of premature labour, first in anthroposophic hospitals and, recently, in conventional settings as an add-on medication. *In vitro* work with hTERT human myometrial cells showed that *B. pinnatum* leaf press juice (BPJ) inhibits the increase of intracellular free calcium concentration ($[Ca^{2+}]_i$) induced by oxytocin (OT), a hormone known to play a role in labour. Our aim was to identify fractions/compounds in BPJ that contribute to this inhibitory effect, and to investigate their effect on OT-driven activation of the MAPK cascade. Several fractions/compounds from BPJ led to a concentration-dependent decrease of OT-induced increase of $[Ca^{2+}]_i$, but none of them was as strong as BPJ. However, the combination of a bufadienolide (BEF) and a flavonoid-enriched fraction (FEF) was as effective as BPJ, and their combination had a synergistic effect. BPJ inhibited OT-driven activation of MAPKs SAPK/JNK and ERK1/2, an effect also exerted by BEF. The effect of BPJ on OT-induced signalling pathways was comparable to that of the OT-receptor antagonist and tocolytic agent atosiban. Our findings further substantiate the use of BPJ preparations in the treatment of preterm labour.

Keywords: *Bryophyllum pinnatum*, myometrium cells, oxytocin, MAPK, intracellular calcium, cell signalling

47 ## Introduction

48 Every year, around 15 million neonates worldwide are born too early. Prematurity
49 (i.e. birth before 37 weeks of pregnancy) is the number one cause of neonatal
50 deaths, and the leading cause of death in children under 5 years of age[1-3]. A number
51 of pharmacological agents known as tocolytics – including beta-sympathomimetic
52 drugs, oxytocin receptor (OTR) antagonists, and calcium channel inhibitors – have
53 been introduced for inhibiting preterm uterine contractions that are responsible for a
54 considerable part of preterm births[4,5]. Tocolysis is usually performed for 48h to allow
55 corticosteroid administration to the mother in order to achieve foetal lung maturation,
56 and *in utero* transfer to a perinatal centre[5,6]. However, to date there are no fully
57 satisfactory tocolytics as often birth still occurs prematurely[7,8]. Moreover, treatment
58 with the usual tocolytic agents is often accompanied by various, in part serious side-
59 effects. These include tachycardia, dyspnoea, palpitation, pulmonary oedema, and
60 hyperglycaemia (in the case of the sympathomimetic drugs)[4], nausea, dizziness,
61 headache, and tachycardia (OTR antagonists)[9] and flushing, hypotension, and
62 suppression of heart rate (calcium channel blockers)[10,11].
63 The beginning of human parturition is clinically manifested by rhythmic uterine
64 contractions leading to the expulsion of the baby. Oxytocin (OT) is a potent
65 physiological stimulator of myometrial contractions, and its receptor and the
66 downstream signalling pathways are attractive targets for drug development aimed at
67 managing preterm labour[4]. At the cellular level, the mechanism by which OT leads to
68 stimulation of the uterus is very complex[12]. Binding of OT to its receptor (OTR) leads
69 to OTR coupling with $G_{\alpha q/11}$ G protein[13] promoting myometrial contractions through
70 multiple signalling pathways[14,15]. $G_{\alpha q/11}$ signalling activates phospholipase C-β (PLC-
71 β), which in turn hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol

72 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 leads to release of calcium
73 ions (Ca^{2+}) from the sarcoplasmic reticulum (SR) into the cytoplasm, and DAG
74 activates protein kinase type C (PKC). A high Ca^{2+}-concentration in the cytoplasm
75 promotes myometrial contraction through activation of myosin light chain kinase
76 (MLCK), known as canonical pathway. PKC activates the mitogen-activated protein
77 kinase (MAPK) cascade[14,15], resulting in increased phospholipase A_2 (PLA_2) activity
78 and prostaglandin E_2 (PGE_2) production which also contributes to contraction[14]. In
79 recent years, the importance of inflammatory processes in labour at term and
80 preterm became apparent[16]. The main MAPKs are the extracellular signal-regulated
81 kinases (ERKs) 1 and 2 (ERK1/2), stress-activated protein kinase or c-Jun amino-
82 terminal kinases (SAPK/JNK) and p38[17,18]. Due to the direct impact of OT on
83 myometrium contractility, OTRs are attractive targets[19]. A widely used competitive
84 inhibitor of the OTR is atosiban[9,20].
85 *Bryophyllum pinnatum* (Lam.) Oken, [syn. *Kalanchoe pinnata* (Lam.), Pers.; family
86 Crassulaceae] has been widely used in traditional medicine of tropical countries, in
87 the treatment of wounds, diabetes mellitus, joint pain, headache, etc[21,22]. In 1970, *B.*
88 *pinnatum* was introduced in obstetrics at the anthroposophic Herdecke Community
89 Hospital (Germany) for the treatment of preterm labour (PTL)[23]. In Switzerland,
90 products containing press juice of *B. pinnatum* leaves are nowadays prescribed for
91 the same indication[24,25]. Several clinical studies have shown a very good tolerability
92 of *B. pinnatum* preparations for this indication[21,22,24,26,27]. *In vitro* studies showed that
93 *B. pinnatum* reduces the strength of human myometrium contractions[28-31]. In human
94 myometrial cells (hTERT-C3 cell line), leaf press juice of *B. pinnatum* (BPJ) lowered
95 the OT-induced increase of intracellular calcium concentrations ($[Ca^{2+}]_i$)[32].

96 Previous phytochemical studies on *B. pinnatum* showed that flavonoid glycosides
97 and bufadienolides are the major classes of secondary metabolites in leaves[33,34]. The
98 presence of flavonoid glycosides (derivatives of quercetin, myricetin, diosmetin, and
99 kaempferol)[35] and bufadienolides (bersaldegenin-1-acetete, bryophyllin A,
100 bersaldegenin-3-acetate, and bersaldegenin-1,3,5-orthoacetate (BO)) was shown[34].
101 We here investigated the inhibitory effects of *B. pinnatum* press juice compounds on
102 OT-induced intracellular signalling, with focus on intracellular calcium levels and
103 activation of MAPK proteins. The effects were compared with those of the OTR-
104 antagonist and tocolytic agent atosiban.
105

106 Materials and methods

107 Cell culture

108 Human myometrial telomerase reverse transcriptase cells (hTERT-C3)[36,37], provided
109 by M. Grãos (Biocant, Cantanhede, Portugal), were cultured in an 1:1 mixture of
110 DMEM and F-12 supplemented with antibiotics (100 U/mL penicillin and 100 µg/mL
111 streptomycin) and 10% (v/v) heat-inactivated foetal bovine serum (FBS) (all from
112 Gibco). Pregnant human myometrial cells (PHM1-41, ATCC® CRL-3046™) were
113 maintained in ATCC-formulated DMEM (ATCC® No. 30-2002) supplemented with
114 10% (v/v) heat inactivated FBS, 2 mM glutamine (Gibco), and 0.1 mg/mL G-418 (Carl
115 Roth).
116

117 Plant material

118 Plant material of *B. pinnatum* originated from two different harvests. Weleda Brazil
119 provided leaves harvested on 25 March 2014 in S. Roque, Brazil. A voucher
120 specimen (ZSS 29717) was deposited at The Zurich Succulent Plant collection.

121 Immediately after the collection, leaves were sent by airmail in a refrigerated
122 container to Weleda AG, Arlesheim, Switzerland. In addition, the Weleda branch
123 located in Schwäbisch Gmünd, Germany, provided leaves harvested in July and
124 August 2010. A voucher specimen (ZSS 29715) was deposited at the Zurich
125 Succulent Plant collection.
126 Plant material of *B. daigremontianum* was provided by the Ita Wegman Hospital
127 Arlesheim, Switzerland, in September 2010. A voucher specimen (No. 838) was
128 deposited at the Division of Pharmaceutical Biology, University of Basel.
129

130 ***Bryophyllum pinnatum* leaf press juice (BPJ)**

131 BPJ was prepared from leaves harvested in S. Roque, Brazil. The press juice was
132 obtained by mechanical pressing of leaves in a roller mill. The procedure was
133 identical to the initial steps of the protocol used for the production of the active
134 ingredient of Weleda Bryophyllum 50% chewing tablets (Weleda AG, Arlesheim,
135 Switzerland). The amount of bufadienolides and flavonoids in BPJ was 0.012 and
136 0.072 mg/mL (based on the determined content of the flavonoid aglycones – 0.034
137 mg/mL), respectively[30,33]. The suspension was filtered using a 150 mm diameter
138 paper filter (Schleicher & Schuell, Dassel, Germany), and aliquots were kept at -80°C
139 until use.
140

141 **Bufadienolide and flavonoid-enriched fractions (BEF and FEF)**

142 Enriched fractions originated from earlier studies[35,38]. Frozen fresh leaves (Weleda,
143 Schwäbisch Gmünd) were lyophilised, powdered in a mortar, and extracted with
144 MeOH. The MeOH extract was partitioned between H_2O and CH_2Cl_2. The aqueous
145 phase was further fractionated by column chromatography (with Diaion HP20 resin)

146 and, after a first elution with H_2O to remove the highly polar compounds, FEF was
147 obtained by elution with MeOH. Evaporation of the CH_2Cl_2-soluble phase yielded a
148 residue[38] that was further purified to afford BEF[33]. The amount of flavonoids in FEF
149 was estimated to be approx. 8.28% based on the determined content of the flavonoid
150 aglycones (4.14%) while the amount of bufadienolides in BEF was found to be
151 9.10%[33,35]. BEF and FEF stock solutions (1.3 and 10.0 mg/mL, respectively) were
152 prepared in DMSO.
153
154 **Flavonoid aglycon mix (A-Mix)**
155 After hydrolysis of FEF, a content of 4.1% of total flavonoid aglycons was
156 determined, and relative proportions were 74.6% of quercetin, 16.7% of myricetin,
157 4.6% of diosmetin, and 4.0% of kaempferol[35]. A mixture of the four aglycons in these
158 proportions (A-Mix) was prepared in DMSO at a concentration of 0.4 mg/mL.
159
160 **Bersaldegenin-1,3,5-orthoacetate (BO)**
161 The compound was previously isolated from *B. daigremontianum*[34]. The amount of
162 BO in BPJ was 0.002 mg/mL[33]. A stock of 0.023 mg/mL was prepared in DMSO.
163
164 **Drugs, reagents and test substances**
165 OT and Digitonin were obtained from Sigma-Aldrich (St. Louis, USA). Fura-2 and
166 Pluronic F-127 were purchased from Molecular Probes-Invitrogen. Atosiban
167 (Tractocile®, 7.5 mg/mL injectable solution) was purchased from Ferring
168 Pharmaceuticals (Baar, Switzerland) and Dimethyl sulfoxide (DMSO) from Sigma
169 (France).

170 All substances tested were diluted in Krebs solution or cell media prior to
171 experiments being performed. The DMSO concentration in test substances was
172 adjusted to 0.1% in calcium experiments and 0.2% in phosphorylation experiments.
173 Control wells were treated to contain the same concentration of DMSO.
174
175 **Measurement of intracellular calcium levels**
176 hTERT-C3 ($8x10^4$ cell/mL) and PHM1-41 ($10x10^4$ cell/mL) cells were seeded into 96-
177 well black microplates (Corning Inc.) two days before experiments were performed.
178 Measurement of intracellular calcium levels was performed as previously described
179 with some adaptations[32]. Briefly, cells were loaded with 10 µM Fura-2/AM
180 reconstituted in DMSO as a 1mM stock solution and 0.06% (w/v) Pluronic F-127 in
181 fresh medium. After 1 h incubation at 37°C, Fura-2 was replaced by fresh medium
182 and cells were incubated for 30 min. Thereafter, cells were washed twice with 100 µL
183 sodium salt solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM
184 Glucose, 10 mM HEPES-Na^+, pH=7.4). Test substances were added in fresh sodium
185 salt solution and fluorescence was read for 4 min, followed by stimulation with 100nM
186 OT (4 min reading). At the end of the experiments, cells were permeabilised with 200
187 µM digitonin followed by Tris-EGTA solution (Tris 1M, EGTA 200 mM, pH=10.2).
188 Fluorescence was read for 2 min. For each substance tested, 4 (PHM1-41 cell line)
189 to 6 (hTERT-C3 cell line) independent cultures, carried out in quadruplicate, were
190 used. A negative control, with no test substance added (or DMSO) to the culture
191 medium and a positive control (5000 nM atosiban) were included in each plate.
192 Fluorescence was measured at emission of 510 nm by illuminating the cells with an
193 alternating 340/380 nm light every 4 s, using a microplate fluorescence reader
194 (EnVision Multilabel Reader, Perkin Elmer). Fluorescence intensities were acquired

195 using the Wallac EnVision Manager software. The relative fluorescence units
196 readings were converted to $[Ca^{2+}]_i$ values (in nM) using the following formula:

197 $$[Ca^{2+}]_i = Kd \times Q \frac{R - R_{min}}{R_{max} - R'}$$

198 Where: Kd = dissociation constant of the Ca^{2+}/Fura-2 complex (224 nM); Q =
199 F_{min}/F_{max} at 380 nm (F_{max} after digitonin and F_{min} after EGTA); R = $F_{340\ nm}/F_{380\ nm}$ (F –
200 fluorescence intensity); R_{max} at maximum Ca^{2+} concentration (after digitonin) and R_{min}
201 at minimum Ca^{2+} concentration (after Tris-EGTA).
202 The variation of intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$) in each well was calculated
203 by subtracting basal readings from the peak of $[Ca^{2+}]_i$ after stimulation with OT (5
204 highest points). The $\Delta[Ca^{2+}]_i$ was normalised to control values.
205 To characterise the effect of combinations, the median-effect method was used[39,40].
206 This method is based on the mass action law and allows a quantitative definition of
207 the interaction between two different drugs. The combination Index (CI) is widely
208 used to asses both beneficial and adverse interactions between pharmaceuticals. CI
209 quantitatively determines/simulates a measure of the extent of drug combination at
210 all doses and all effects with small number of data points[39,40]. CI is calculated
211 according to which the interaction between two drugs can be classified as
212 antagonistic (CI>1), additive (CI=1) or synergistic (CI<1). CI values were calculated
213 using Compusyn software.
214

215 **Phosphorylation experiments**

216 hTERT-C3 (4.7×10^4 cell/mL) were seeded into 6-well plates three to four days before
217 experiments were performed. Once 90% confluence was reached, cells were treated
218 with OT (100 nM) for 2, 5, 15, 30 or 45 min. To investigate the modulation of OT-
219 induced phosphorylation, cells were pre-treated with BPJ (2% corresponding to 20

220 µg/mL), BEF (2.20 µg/mL), FEF (17.39 µg/mL), A-Mix (0.68 µg/mL), different
221 combinations, atosiban (100 nM) or just medium for 30 min before stimulation with
222 OT (time 0 of OT-incubation). Stimulation was stopped by washing with ice-cold PBS,
223 and plates were flash frozen in liquid nitrogen. Afterwards, cells were lysed in
224 radioimmunoprecipitation assay (RIPA) buffer and phosphatase and protease
225 inhibitor cocktail (both from Thermo scientific).
226 Proteins (20 µg) were separated in a 12% SDS polyacrylamide gel, and transferred
227 to a PVDF membrane. When protein samples from one experiment were run in more
228 than one gel, a normalisation sample (NS) constituted by proteins extracted from
229 hTERT-C3 cells exposed to OT for 5 min was used in all gels to decrease variability
230 between blots. Membranes were incubated in primary antibody: GAPDH, phospho-
231 p38 (Thr180/Tyr182), phospho-p44/42 ERK1/2 (Thr202/Tyr204), phospho-SAPK/JNK
232 (Thr183/Tyr185) overnight at 4°C and in the appropriated Horseradish peroxidase
233 (HRP)-conjugated secondary antibody (all from Cell Signalling) for 1h at room
234 temperature the next day. Signal detection was done using Supersignal™ West Pico
235 Plus Chemiluminescent Substrate (Thermo scientific). Detection and quantification of
236 band intensities was performed using FusionCapt Advance system (Vilber,
237 Germany). Equal loading was confirmed by blotting the membranes for GAPDH. For
238 each substance tested, 4 independent cultures of hTERT-C3 cell line were used.
239

240 **Statistical analysis**

241 All results were expressed as mean ± standard error of the mean (SEM) and
242 statistical analyses were performed with Graphpad Prism software. The Shapiro-Wilk
243 test was used to check normal distribution. For intracellular calcium measurements
244 one-way ANOVA with Dunnett's *post-hoc* test was conducted to compare different

245 concentrations of each test substance to the corresponding control. Ordinary two-
246 way ANOVA was used to compare different dose-dependency treatments. For
247 phosphorylation study, RM two-way ANOVA with Dunnett's multiple comparison was
248 used to evaluate differences between each time point to time 0 min, and to OT-
249 treated. To compare the different test substances to non-stimulated control or to OT-
250 stimulated cells, the Mann-Whitney was used. Values were considered to be
251 statistically significant if $p<0.05$.
252

253 ## Results

254 **Inhibition of OT-induced rise of $[Ca^{2+}]_i$ by BPJ fractions/compounds**

255 We previously showed that BPJ inhibits OT-induced rise of $[Ca^{2+}]_i$[32]. To investigate
256 the contribution of different constituents/fractions of *B. pinnatum* on the OT-induced
257 rise of $[Ca^{2+}]_i$, hTERT-C3 cells were pre-incubated with FEF, BEF, A-Mix and BO.
258 The $\Delta[Ca^{2+}]_i$ decreased progressively and in a statistically significant way when cells
259 were pre-incubated with FEF and A-Mix (FEF: $p=0.043$, A-Mix: $p=0.0008$). Compared
260 to control, the values of $\Delta[Ca^{2+}]_i$ were significantly lower at 4.35 µg/mL of FEF
261 ($p=0.030$) and 0.17 µg/mL of A-Mix ($p=0.014$; Fig. 1a).
262 As shown in Fig. 1b, the effect of BEF and BO on the OT-induced rise of $[Ca^{2+}]_i$ was
263 concentration dependent (BEF: $p=0.0001$, BO: $p=0.022$). When each concentration
264 was compared to control, a significant difference was obtained with 0.55 µg/mL of
265 BEF ($p=0.01$) and 0.035 µg/mL of BO ($p=0.028$). Under our experimental conditions,
266 none of the fractions/compounds had an effect that was equally strong as that of BPJ
267 (Figs. 1a and 1b).
268 Concentrations of FEF, BEF, A-Mix and BO used during the experiments are
269 correspondent to those found in BPJ (Fig. 1c).

270

271 **Inhibition of OT-induced rise of $[Ca^{2+}]_i$ by combinations of BEF with FEF, and of**

272 **BEF with A-Mix**

273 To assess whether more than one fraction/compounds mixture was needed to obtain

274 an effect comparable to that of BPJ, combinations of BEF with FEF, and of BEF with

275 A-Mix were investigated, again at test concentrations that corresponded to their

276 content in BPJ. BEF plus FEF led to a concentration-dependent inhibition of $\Delta[Ca^{2+}]_i$,

277 whereby all concentrations were significantly different from control ($p<0.0001$). No

278 statistically significant difference was observed when comparing the effect of BEF

279 plus FEF with that of BPJ. The combination of the highest concentrations of BEF and

280 FEF (2.20 and 17.39 µg/mL, respectively) was significantly different from each

281 substance alone at the same concentrations (BEF: $p=0.030$, FEF: $p=0.009$; Fig. 2a).

282 Pre-incubation of cells with the combination of BEF plus A-Mix also showed a

283 concentration-dependent effect ($p<0.0001$) on the OT-induced rise of $[Ca^{2+}]_i$, and at

284 all test concentrations the effect was significantly different from control. However, the

285 effect of the combination was generally weaker than that of BPJ. The combination of

286 the highest concentrations of BEF and A-Mix (2.20 and 0.68 µg/mL, respectively)

287 was significantly different from BPJ ($p=0.0035$; Fig. 2c).

288

289 **Characterisation of the combined effects**

290 The median-effect method was used to analyse the data for antagonism, additivity or

291 synergism of the combinations. Figures 2b and 2d show that combination index (CI)

292 values less than 1 were obtained with the combinations studied, which is indicative

293 for synergistic interaction. Fraction affected (Fa) values for the various combinations

294 of BEF and FEF ranged between 0.2 to 0.6, indicating that the synergistic interaction

295 was observed when the inhibition of OT-induced increase of intracellular Ca^{2+} levels
296 was 20 to 60% (Fig. 2b). Fa values for the combination of BEF and A-Mix ranged
297 between 0.2 to 0.4, reflecting a weaker maximal inhibition by this combination (Fig.
298 2d).
299

300 **BPJ inhibits OT-induced rise of $[Ca^{2+}]_i$ in PHM1-41 myometrial cells**

301 As previously shown, the exposure of hTERT-C3 cells to 100 nM OT induced an
302 increase of $[Ca^{2+}]_i$ with a peak response at about 10 to 20 sec after stimulation, and a
303 subsequent decrease to resting conditions (Fig. 3a and 3b). Pre-incubation with BPJ
304 (0.1%-2.0% corresponding to 1.0-20.0 µg/mL) led to a concentration dependent
305 decrease of the $[Ca^{2+}]_i$ peak induced by OT ($p<0.0001$; Fig. 3a). To verify if this effect
306 was cell line dependent, experiments were conducted in a second myometrial cell
307 line (PHM1-41). A peak response of $[Ca^{2+}]_i$ was observed at 12 to 20 sec after
308 stimulation with OT (Fig. 3c and 3d). When pre-incubated with BPJ, a decrease of
309 the OT-induced increase of cytosolic $[Ca^{2+}]_i$ peak was observed (Fig. 3c). BPJ thus
310 promoted a concentration-dependent effect on $\Delta[Ca^{2+}]_i$ in both cell lines ($p<0.0001$;
311 Fig. 3e), with significant effects at concentrations >2.5 µg/mL in hTERT-C3 cells
312 ($p=0.012$), and >5.0 µg/mL in PHM1-41 cells ($p=0.026$).
313 In both cell lines, the effect of BPJ was compared with that of the OTR antagonist
314 atosiban. Pre-incubation of hTERT-C3 and PHM1-41 with atosiban led to a decrease
315 of the OT-induced $[Ca^{2+}]_i$ peak (Fig. 3b and 3d, respectively), and promoted a
316 concentration-dependent effect on $\Delta[Ca^{2+}]_i$ ($p<0.0001$, both cell lines; Fig. 3f). The
317 highest concentration of atosiban (5000nM) lowered $\Delta[Ca^{2+}]_i$ to 6.04 and 22.46% of
318 control in hTERT-C3 and PHM1-41 cells, respectively.
319

320 **BPJ inhibits OT-induced phosphorylation of MAPK proteins**

321 We investigated the effect of BPJ on OT-induced phosphorylation of p38, SAPK/JNK
322 and ERK1/2 in hTERT-C3 cells. Time-course experiments revealed that
323 phosphorylation of these three MAPKs increased markedly during the first 5 min of
324 incubation with OT (Figs 4a, c and e). The extent of p38 phosphorylation was
325 constant for further 10 min before starting to decrease (Fig. 4a), whereas SAPK/JNK
326 and ERK1/2 phosphorylation decreased already after 10 min of incubation with OT
327 (Figs 4c and 4e). After 45 min, the levels of phosphorylated p38, SAPK/JNK and
328 ERK1/2 were comparable to basal values.

329 As shown in Fig. 4a, pre-treatment with BPJ delayed, but did not prevent, OT-
330 triggered p38 phosphorylation. BPJ seemed to attenuate the effect of OT on p38
331 activation at 5 min, but the difference did not reach statistical significance. For
332 comparison, atosiban was included in the time-course experiments. This OTR
333 antagonist led to a significant inhibition of OT-induced phosphorylation of p38 at 5
334 and 15 min (p=0.001 and p=0.001; Fig. 4a). The effects of pre-treating the cells with
335 BPJ fractions/compounds alone or combined on OT-induced p38 phosphorylation
336 were comparable to the one of BPJ, i.e., none of the test substances promoted a
337 significant decrease of p38 phosphorylation induced after at 5 min incubation with OT
338 (Fig. 4b).

339 Regarding SAPK/JNK, pre-treatment with BPJ led to a significant decrease of the
340 maximal OT-driven phosphorylation (p=0.0002). A similar inhibition was observed
341 when cells were pre-incubated with atosiban (p=0.0002; Fig. 4c). Pre-treatment with
342 BEF alone and even more BEF plus FEF induced a significant decrease in the
343 maximal amount of OT-induced phosphorylation of SAPK/JNK (in both cases
344 p=0.029). The effect of the combination was similar to the one of BPJ (Fig. 4d).

345 Considering p-ERK1/2, pre-treatment with BPJ significantly reduced the OT-driven
346 maximal activation, which was observed after 5 min incubation (p=0.006). Also, in
347 this case, atosiban reduced maximal activation (p=0.001; Fig. 4e). The results
348 obtained with BPJ fractions/compounds showed that only BEF promoted an inhibition
349 similar to that observed upon pre-treatment with BPJ (p=0.029). None of the other
350 fractions/compounds significantly reduced the OT-driven phosphorylation of ERK1/2
351 (Fig. 4f).
352

353 ## Discussion

354 BPJ appears to inhibit several OT-induced signalling pathways that are involved in
355 the regulation of myometrial contractility. Apart from an inhibition of the OT-induced
356 increase of $[Ca^{2+}]_i$, various fractions obtained from BPJ exhibit similar, albeit weaker,
357 effects. In contrast, the combination of BEF and FEF had an effect on the canonical
358 signalling pathway that is comparable to that of BPJ, and the combination of these
359 fractions enriched in bufadienolides (BEF) and flavonoid glycosides (FEF) is
360 synergistic. Other OT-induced pathway, namely the activation of the MAPKs
361 SAPK/JNK and ERK1/2 is inhibited by BPJ, whereby the bufadienolide fraction
362 seems to be chiefly responsible for the inhibition of ERK1/2 phosphorylation, and the
363 combination of BEF plus FEF for the inhibition of SAPK/JNK phosphorylation. In
364 contrast, activation of p38 was hardly affected. The main findings are depicted in Fig.
365 5.
366 To better understand which compound classes in BPJ might be responsible for its
367 effect on the OT-induced increase of $[Ca^{2+}]_i$, BEF, FEF, A-Mix and BO were tested at
368 concentrations that corresponded to those in BPJ (Fig. 1c). In addition, we studied
369 the effect of two different combinations, namely BEF plus FEF, and BEF plus A-Mix

370 (Fig. 2). The amounts of flavonoid aglycons in BPJ and FEF, and of bufadienolides
371 present in BPJ and BEF have been determined previously [30,33,35], allowing an exact
372 comparison of the various related substances. Since even longer incubations at
373 higher concentrations of BPJ fractions/compounds did not affect cell viability[30], it is
374 likely that the results reflect a true effect on OT-signalling. All fractions/compounds
375 tested promoted a concentration-dependent decrease of OT-induced increase of
376 $[Ca^{2+}]_i$, but these effects were not as strong as that of BPJ (Fig. 1). Only the effect of
377 a combination of BEF and FEF was comparable to that of BPJ. This indicates that
378 several compound classes in BPJ contribute to the inhibition of the OT-induced
379 increase of $[Ca^{2+}]_i$, and that the effects of BEF and FEF (Fig. 2b) are synergistic.
380 Synergy has often been postulated as being important for the pharmacological
381 activity of phytomedicines, but this is one of only a few cases where this has been
382 shown experimentally[41,42].
383 Press juice of *B. pinnatum* leaves is the active ingredient of preparations that are
384 being recommended and used in Switzerland to treat preterm labour, often as an
385 add-on treatment[24,25,43]. Using the myograph model, we previously showed that BPJ
386 concentration-dependently inhibits spontaneous myometrium contractions, affecting
387 contraction peak, tension, and contraction duration[29,30]. Stronger effects on
388 spontaneous contractility were observed when BPJ was combined with known
389 tocolytics such as atosiban and nifedipine[29]. The present work confirms earlier results
390 showing that BPJ inhibits the canonical pathway of OT-induced increase of
391 contractility in hTERT-C3 human myometrium cells[32]. A comparable inhibition of the
392 OT-induced increase of $[Ca^{2+}]_i$ has now been observed in PHM1-41 myometrial cells
393 (Fig. 3e). In both cell lines, pre-treatment with the OT-antagonist atosiban led to an
394 inhibition of the canonical pathway.

395 Flavonoid glycosides are known to be hydrolysed by gut microbiota, leading to
396 release of aglycons[44,45]. In our experimental settings, the effects of FEF and A-Mix
397 were comparable, suggesting that cleavage of the sugar moieties does not affect
398 their activity in the models used. Only in the combination experiments with BEF, A-
399 Mix had weaker effects than FEF. It may be that the glycosides contribute to a
400 stronger synergistic effect, or that FEF contains additional compounds that are
401 relevant for the activity.
402 Early preterm birth is often associated with increased myometrium contractility, but
403 uterus inflammation is also an important risk factor for foetal and neonatal central
404 nervous system damage[46]. Therefore, an ideal tocolytic agent should have both
405 functions. Our results show that BPJ, and in particular the corresponding
406 bufadienolides, prevent the OT-induced phosphorylation of two relevant MAPKs,
407 namely SAPK/JNK and ERK1/2. This suggests that downstream enzymes might also
408 be inhibited and prostaglandin production lowered as a consequence. Whether *B.*
409 *pinnatum* preparations can inhibit inflammatory processes that might lead to
410 increased myometrial contraction and eventually parturition needs further
411 investigation.
412 In the development of new tocolytic agents, a simultaneous inhibition of the
413 immediate, calcium mediated canonical pathway and of the activation of MAPK-
414 dependent pathways in the myometrium is nowadays seen as a required
415 pharmacological profile[15]. The present data show that *B. pinnatum* matches with
416 these requirements, which in turn further substantiates its use in the treatment of
417 preterm labour.
418

419 **Data Availability**

All data generated or analysed during the currently study are available from the corresponding author on reasonable request.

References

[1] WHO, World Health Organization. Preterm birth. Available at http://www.who.int/mediacentre/factsheets/fs363/en/ (2018).

[2] Blencowe, H. *et al.,*. Born too soon: the global epidemiology of 15 million preterm births. *Reprod. Health* **10,** S2 (2013).

[3] Lawn, J. E. *et al.,*. Born too soon: care for the preterm baby. *Reprod health* **10,** S5 (2013).

[4] Arrowsmith, S., Kendrick, A. & Wray, S. Drugs acting on the pregnant uterus. *Obstet, Gynaecol Reprod. Med.* **20,** 241-247 (2010).

[5] Romero, R., Dey, S. K. & Fisher, S. J. Preterm labor: one syndrome, many causes. *Science* **345,** 760-765 (2014).

[6] Schleußner, E. The prevention, diagnosis and treatment of premature labor. *Deutsches Ärzteblatt International* **110,** 227 (2013).

[7] Lamont, C. D., Jorgensen, J. S. & Lamont, R. The safety of tocolytics used for the inhibition of preterm labour. *Expert Opin. Drug Saf.* **15,** 1163-1173 (2016).

[8] Haram, K., Mortensen, J. H. S. & Morrison, J. C. Tocolysis for acute preterm labor: does anything work. *J. Matern Fetal Neonatal Med.* **28,** 371-378 (2015).

[9] Flenady, V., Reinebrant, H. E., Liley, H. G., Tambimuttu, E. G. & Papatsonis, D. N. Oxytocin receptor antagonists for inhibiting preterm labour. *Cochrane Database Syst. Rev.*, CD004452, doi:10.1002/14651858.CD004452.pub2 (2014 Jun 6).

[10] Flenady, V. *et al.,*. Calcium channel blockers for inhibiting preterm labour and birth. *Cochrane Database Syst. Rev.*, CD002255, doi:10.1002/14651858.CD002255.pub2 (2014 Jun 5).

[11] Gáspár, R. & Hajagos-Tóth, J. Calcium channel blockers as tocolytics: principles of their actions, adverse effects and therapeutic combinations. *Pharmaceuticals* **6,** 689-699 (2013).

[12] Aguilar, H. N. & Mitchell, B. Physiological pathways and molecular mechanisms regulating uterine contractility. *Hum. Reprod. Update* **16,** 725-744 (2010).

[13] Phaneuf, S. *et al.,*. Oxytocin-stimulated phosphoinositide hydrolysis in human myometrial cells: involvement of pertussis toxin-sensitive and-insensitive G-proteins. *J. endocrinol.* **136,** 497-NP (1993).

[14] Arrowsmith, S. & Wray, S. Oxytocin: its mechanism of action and receptor signalling in the myometrium. *J. Neuroendocrinol.* **26,** 356-369 (2014).

[15] Kim, S. H., Bennett, P. R. & Terzidou, V. Advances in the role of oxytocin receptors in human parturition. *Mol. Cell. Endocrinol.* **449,** 56-63 (2017).

[16] Kim, S. H. *et al.,*. Oxytocin activates NF-κB-mediated inflammatory pathways in human gestational tissues. *Mol. Cell. Endocrinol.* **403,** 64-77 (2015).

[17] Cuenda, A. & Rousseau, S. p38 MAP-kinases pathway regulation, function and role in human diseases. *BBA-Mol. Cell Res.* **1773,** 1358-1375 (2007).

[18] Roux, P. P. & Blenis, J. J. M. M. B. R. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. **68,** 320-344 (2004).

[19] Gulliver, D. *et al.,*. Targeting the oxytocin system: new pharmacotherapeutic approaches. *Trends Pharmacol. Sci.* **40,** 22-37 (2018).

[20] Hubinont, C. & Debiève, F. Prevention of preterm labour: 2011 update on tocolysis. *J. Pregnancy* **2011** (2011).

[21] Fürer, K., Simões-Wüst, A. P., von Mandach, U., Hamburger, M. & Potterat, O. *Bryophyllum pinnatum* and related species used in anthroposophic medicine: constituents, pharmacological activities, and clinical efficacy. *Planta Med.* **82,** 930-941 (2016).
[22] Hamburger, M., Potterat, O., Furer, K., Simoes-Wüst, A. P. & von Mandach, U. *Bryophyllum pinnatum*-Reverse Engineering of an Anthroposophic Herbal Medicine. *Nat. Prod. Commun.* **12,** 1359-1364 (2017).
[23] Hassauer, W., Schreiber, K. & Von der Decken, D. Ein neuer Weg in der tokolytischen Therapie. *Erfahrungsheikunde* **34,** 683-687 (1985).
[24] Fürer, K. *et al.,*. Die Anwendung von *Bryophyllum pinnatum*-Präparaten in der Geburtshilfe und Gynäkologie-eine multizentrische prospektive Beobachtungsstudie. *Complement. Med. Res.* **22,** 231-236 (2015).
[25] Schenkel, L., Simões-Wüst, A. P., Hoesli, I. & von Mandach, U. Drugs in pregnancy and lactation - medications used in Swiss obstetrics. *Z. Geburtshilfe Neonatol.* **222,** 1-14 (2018).
[26] Plangger, N., Rist, L., Zimmermann, R. & von Mandach, U. Intravenous tocolysis with *Bryophyllum pinnatum* is better tolerated than beta-agonist application. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **124,** 168-172 (2006).
[27] Simões-Wüst, A. P. *et al.,*. Two randomised clinical trials on the use of *Bryophyllum pinnatum* in pre-term labour – results after discontinuation. *Complement. Med. Res.*, doi:10.1159/000487431 (2018).
[28] Gwehenberger, B., Rist, L., Huch, R. & von Mandach, U. Effect of *Bryophyllum pinnatum* versus fenoterol on uterine contractility. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **113,** 164-171 (2004).
[29] Santos, S. *et al.,*. Bryophyllum pinnatum enhances the inhibitory effect of atosiban and nifedipine on human myometrial contractility: an in vitro study. *BMC Complement. Altern. Med.* **19,** 292 (2019).
[30] Santos, S. *et al.,*. A Bufadienolide-Enriched Fraction of Bryophyllum pinnatum Inhibits Human Myometrial Contractility In Vitro. *Planta Med.* **85,** 385-393, doi:10.1055/a-0810-7704 (2019).
[31] Wächter, R. *et al.,*. Leaf press juice from *Bryophyllum pinnatum* (Lamarck) Oken induces myometrial relaxation. *Phytomedicine* **19,** 74-82 (2011).
[32] Simões-Wüst, A. P. *et al.,*. Juice of *Bryophyllum pinnatum* (Lam.) inhibits oxytocin-induced increase of the intracellular calcium concentration in human myometrial cells. *Phytomedicine* **17,** 980-986 (2010).
[33] Oufir, M. *et al.,*. Quantification of bufadienolides in *Bryophyllum pinnatum* leaves and manufactured products by UHPLC-ESIMS/MS. *Planta Med.* **81,** 1190-1197 (2015).
[34] Fürer, K. *et al.,*. Two new flavonol glycosides and a metabolite profile of *Bryophyllum pinnatum*, a phytotherapeutic used in obstetrics and gynaecology. *Planta Med.* **79,** 1565-1571 (2013).
[35] Bachmann, S. *et al.,*. Potential of *Bryophyllum pinnatum* as a detrusor relaxant: an in vitro exploratory study. *Planta Med.* **83,** 1274-1280 (2017).
[36] Condon, J. *et al.,*. Telomerase immortalization of human myometrial cells. *Biol. Reprod.* **67,** 506-514 (2002).
[37] Devost, D. & Zingg, H. H. Novel in vitro system for functional assessment of oxytocin action. *Am. J. Physiol-Endoc Metab.* **292,** E1-E6 (2007).
[38] Fürer, K. *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. *Phytomedicine* **22,** 158-164 (2015).
[39] Chou, T.-C. & Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzym Regul.* **22,** 27-55 (1984).

[40] Chou, T.-C. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res.* **70,** 440-446 (2010).
[41] Butterweck, V. & Nahrstedt, A. What is the best strategy for preclinical testing of botanicals? A critical perspective. *Planta Med.* **78,** 747-754 (2012).
[42] Wagner, H. Synergy research: approaching a new generation of phytopharmaceuticals. *Fitoterapia* **82,** 34-37 (2011).
[43] Simões-Wüst, A. P., Nietlispach, A., Mennet, M. & von Mandach, U. *Bryophyllum pinnatum* use in tocolysis in a conventional setting: a retrospective analysis from obstetric clinical practice. *BMC Complement. Altern. Med.* **17 (Suppl 1),** 136 (2017).
[44] Cassidy, A. & Minihane, A.-M. The role of metabolism (and the microbiome) in defining the clinical efficacy of dietary flavonoids. *Am. J. Clin. Nutr.* **105,** 10-22, doi:10.3945/ajcn.116.136051 (2017).
[45] Braune, A. & Blaut, M. Bacterial species involved in the conversion of dietary flavonoids in the human gut. *Gut microbes* **7,** 216-234 (2016).
[46] Romero, R. *et al.,*. The preterm parturition syndrome. *BJOG: Int. J. Obstet. Gynaecol.* **113,** 17-42 (2006).

Acknowledgements

The authors are grateful to Prof. Dr. med A. Aguzzi for allowing the use of his facilities and Dr. G. Chafik for valuable advices and shared expertise. We are indebted to the members of the Bryophyllum Study Group and of the Department of Obstetrics from the University Hospital Zurich for interesting discussions, and to Karin Fürer for preparation of FEF and of a bufadienolide-enriched fraction that was eventually purified to obtain BEF. Financial support was provided by Weleda AG and the Johannes Kreyenbühl Foundation.

Author Contributions

SS and APSW designed the study. SS, with the help of LZ, performed the experiments. SS analysed the data and wrote the first version of the manuscript. APSW, MH, UM, OP and MM were involved in the interpretation of data and provided critical revision of the manuscript. All authors were closely involved in revising the article and agreed with the final version.

547

548 ## Additional information

549 **Competing interests:**

550 MM is an employee of Weleda AG, the company that produces the preparations of
551 *Bryophyllum pinnatum*. APSW received research funding from Weleda AG during the
552 last 5 years. SS, LZ, MH, UM, OP declare no competing interests.

553

554 ## Figure Legends

555 **Figure 1.** Concentration-dependent effects of BPJ fractions/compounds on the OT-
556 induced increase of $[Ca^{2+}]_i$. Myometrial hTERT-C3 cells were pre-treated with FEF or
557 A-Mix (**a**) and BEF or BO (**b**) prior to stimulation with 100 nM of OT. Results of a pre-
558 treatment with BPJ are shown in transparent line. Values represent the mean ± SEM
559 of 6 independent experiments performed in quadruplicate and are expressed as
560 percentage of control; *$p<0.05$. In **c,** the concentrations of FEF, BEF, A-Mix and BO
561 corresponding to the BPJ concentrations are shown.

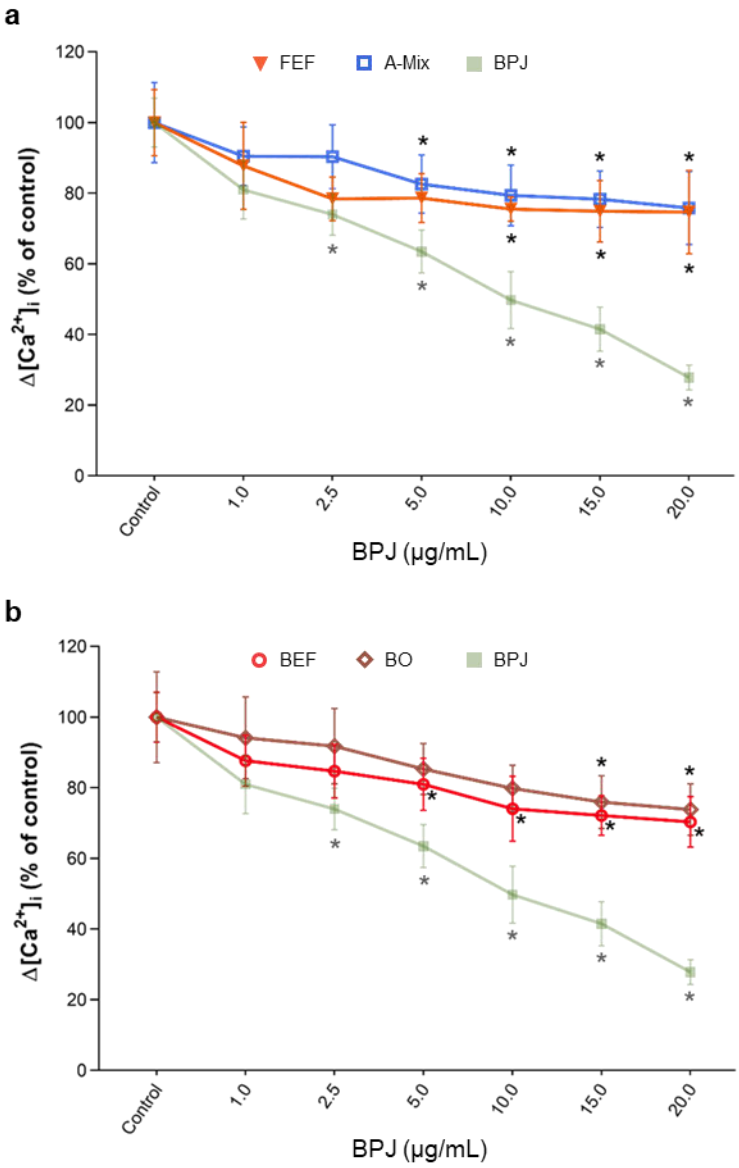
562

563 **Figure 2.** Effect of the combination of BPJ fractions/compounds on the OT-induced
564 rise of $[Ca^{2+}]_i$. Cells were pre-treated with BEF plus FEF (**a**), or with BEF plus A-Mix
565 (**c**) prior to stimulation with 100 nM OT. Results of pre-treatment with BPJ and each
566 substance alone are shown in transparent lines (significance symbols regarding
567 comparison to control omitted). Values represent the mean ± SEM of 6 independent
568 experiments performed in quadruplicate and are expressed as percentage of control;
569 *$p<0.05$ compared with control; $^{\#}p<0.05$ compared to combination. Combination
570 index (CI) values of the combination of BEF with FEF (**b**) or with A-Mix (**c**) were

571 calculated from the concentration-response curves. Data were analysed by the
572 median-effect method. Fa: fraction affected.
573
574 **Figure 3.** Comparison between the effects of BPJ and atosiban on OT-induced rise
575 of $[Ca^{2+}]_i$ in myometrial cell lines. Time-course of OT-induced $[Ca^{2+}]_i$ response in
576 hTERT-C3 (**a** and **b**) and PHM1-41 (**c** and **d**) cells when pre-incubated in the
577 absence (**a-d**, grey lines) or in the presence of BPJ (**a** and **c**) or atosiban (**b** and **d**).
578 $[Ca^{2+}]_i$ was measured for 4 min before stimulation with 100 nM OT. Data shown are
579 from one representative experiment. Concentration-dependent effect of BPJ (**e**) or
580 atosiban (**f**) on the oxytocin-induced $[Ca^{2+}]_i$ increase in hTERT-C3 (lighter colour) and
581 PHM1-41 (darker colour) cells. Values represent the mean ± SEM of 4 (PHM1-41) or
582 6 (hTERT-C3) independent experiments performed in quadruplicate and are
583 expressed as percentage of control; *$p<0.05$.
584
585 **Figure 4.** Effect of BPJ fractions/compounds and atosiban on OT-induced MAPKs
586 phosphorylation. In the time-course experiments, hTERT-C3 cells were pre-treated
587 with or without 20 µg/mL BPJ or 100 nM atosiban for 30 min, before incubation with
588 100 nM OT for 2, 5, 15, 30, and 45 min (**a**, **c** and **e**). To compare the effects of BPJ
589 and the various fractions/compounds, cells were pre-treated with 20 µg/mL BPJ, 2.20
590 µg/mL BEF, 17.39 µg/mL FEF, 0.68 µg/mL A-Mix, BEF plus FEF, or BEF plus A-Mix
591 (same concentrations as for single fractions) for 30 min before stimulation with OT for
592 5 min (**b**, **d** and **f**). Whole cell proteins were subjected to western blot analysis with
593 antibodies against phosphorylated p38 (**a** and **b**), SAPK/JNK (**c** and **d**) and ERK1/2
594 (**e** and **f**); matching densitometry analyses are depicted bellow the representative
595 blots. Samples from the same experiment were processed in parallel and

596 membranes were probed with GAPDH to confirm equal loading. Blot images are from
597 a representative experiment, and line between bands delineate boundary between
598 the gels that were cropped. Full-length blots/gels are presented in Supplementary
599 Figure S1. Values represent the mean ± SEM of 4 independent experiments; *$p<0.05$
600 compared with control; $^{\#}p<0.05$ compared to OT-treated.
601
602 **Figure 5.** Schematic representation of the effects of BPJ fractions on the OT-induced
603 signalling pathways. Myometrial contraction is induced by OT via activation of several
604 intracellular signalling pathways, such as the canonical and the MAPKs pathways.
605 Taken together, our data indicate that BPJ and the combination of BEF and FEF
606 inhibit the OT-induced increase of $[Ca^{2+}]_i$. In what concerns the MAPK pathways, BPJ
607 prevents phosphorylation of SAPK/JNK and ERK1/2, whereby the combination of
608 BEF and FEF seems to be needed for a strong inhibition of SAPK/JNK
609 phosphorylation, while BEF alone appears to be sufficient to preclude ERK1/2
610 phosphorylation.
611

612 Figure 1.



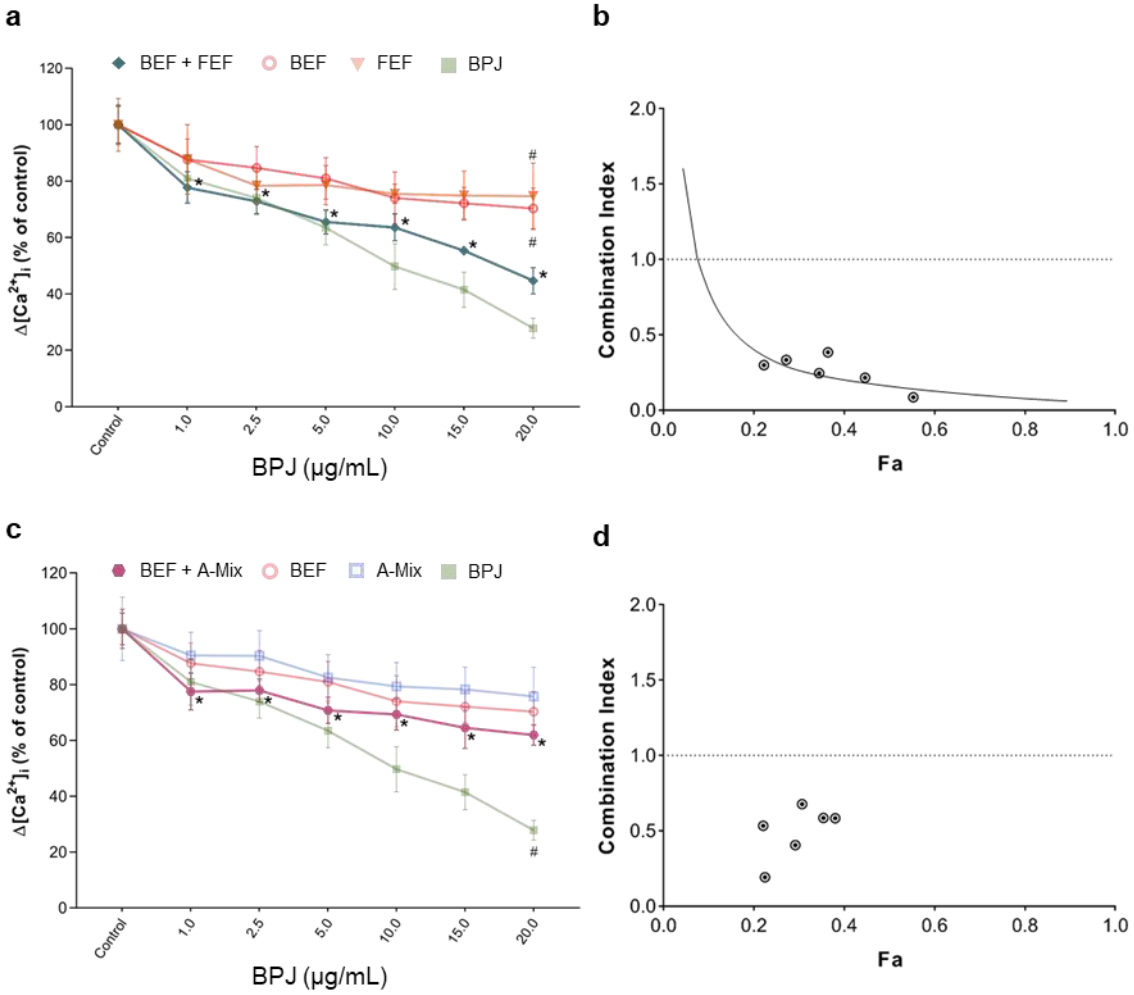
c

BPJ (μg/mL)	1.0	2.5	5.0	10.0	15.0	20.0
FEF (μg/mL)	0.87	2.17	4.35	8.70	13.04	17.39
A-Mix (μg/mL)	0.03	0.09	0.17	0.34	0.51	0.68
BEF (μg/mL)	0.11	0.27	0.55	1.10	1.65	2.20
BO (μg/mL)	0.002	0.005	0.010	0.020	0.030	0.040

613

614

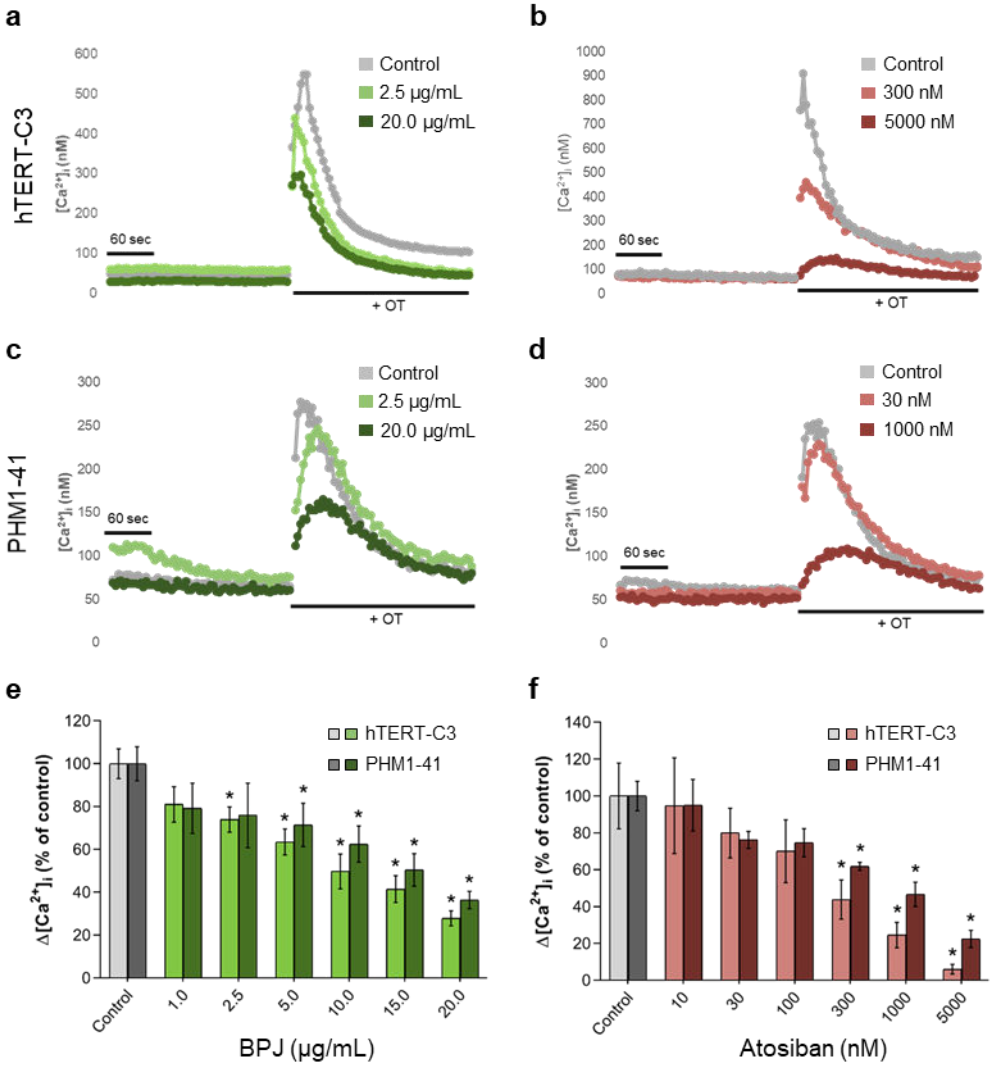
615 Figure 2.



616

617

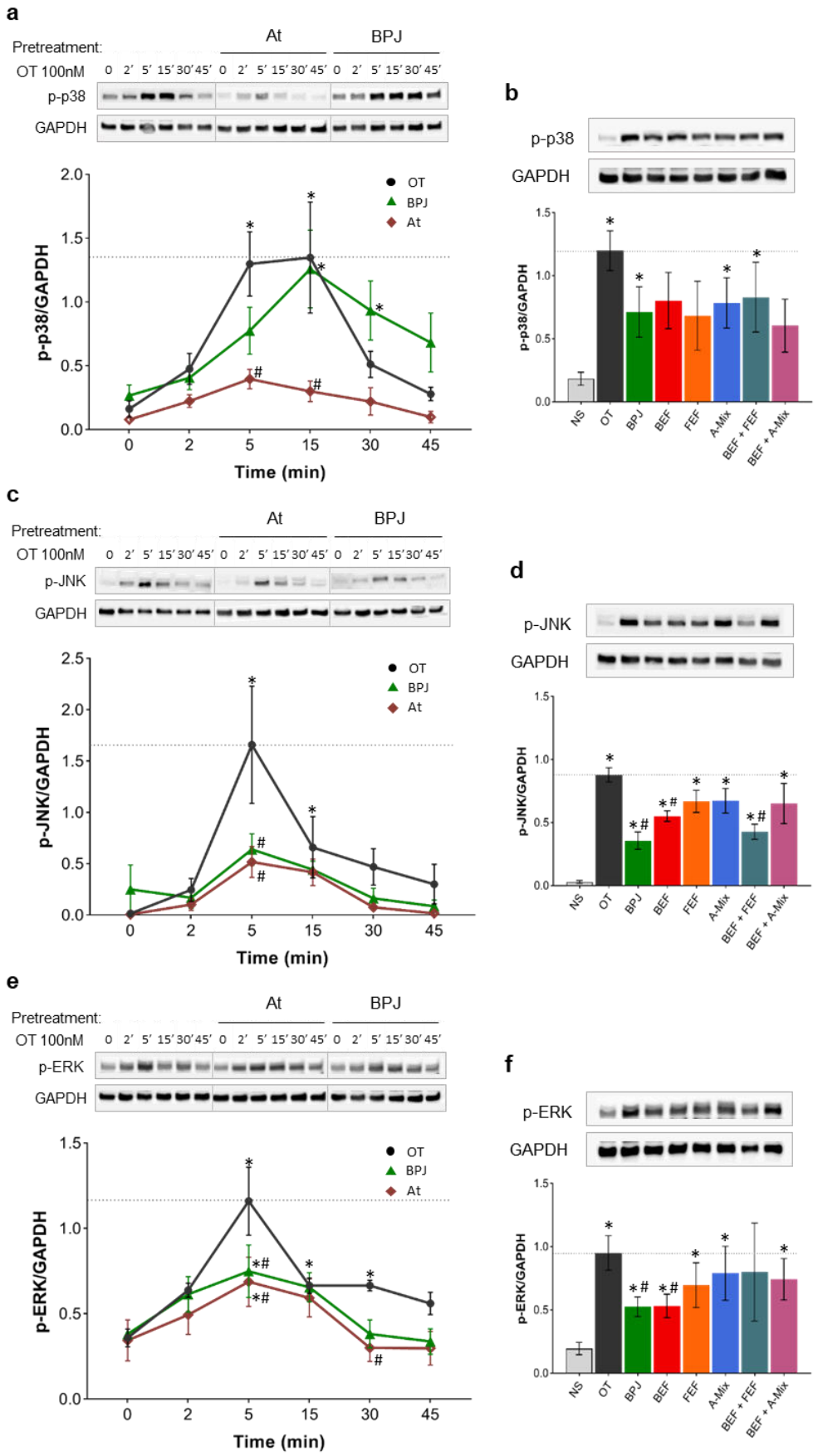
618 Figure 3.



619

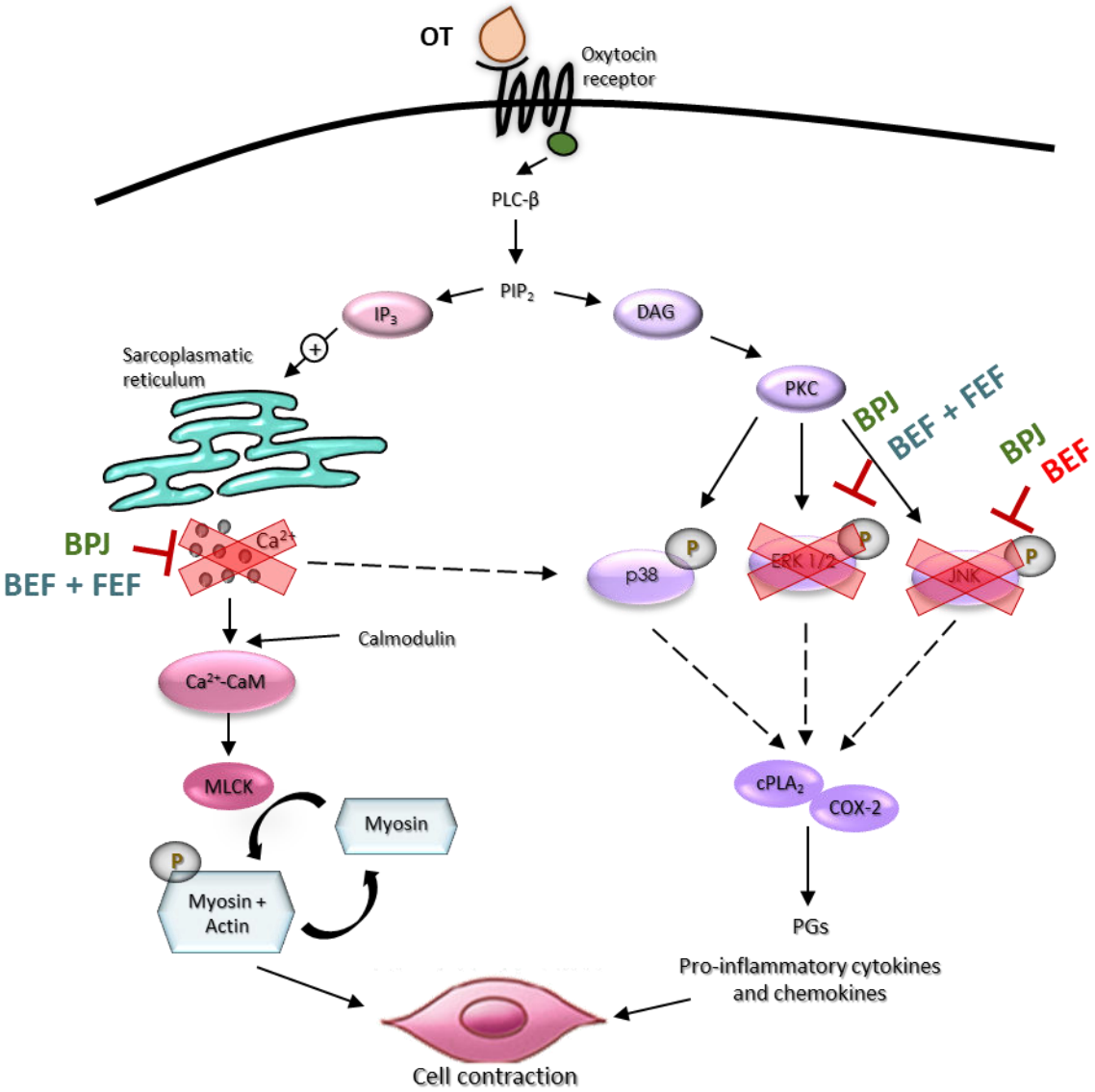
620

621 Figure 4.



622

623 Figure 5.



624

Supplementary information

Bryophyllum pinnatum compounds inhibit oxytocin-induced signalling pathways in human myometrial cells

S. Santos[1,2], L. Zurfluh[1], Mónica Mennet[3], O. Potterat[2], U. von Mandach[2], M. Hamburger[2], A.P. Simões-Wüst[1*]

[1]Department of Obstetrics, University Hospital Zurich, Zurich, Switzerland

[2]Division of Pharmaceutical Biology, University of Basel, Basel, Switzerland

[3]Weleda AG, Arlesheim, Switzerland

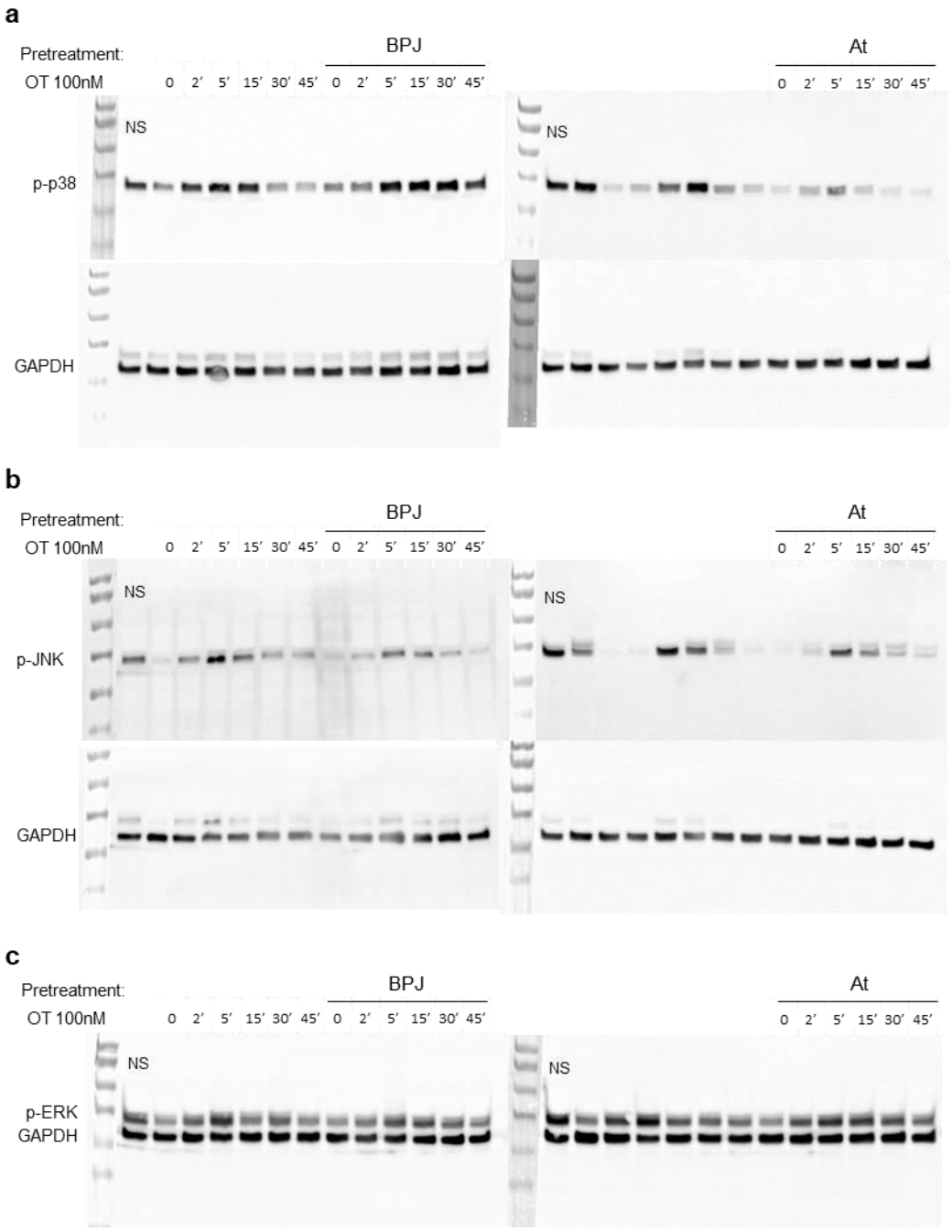
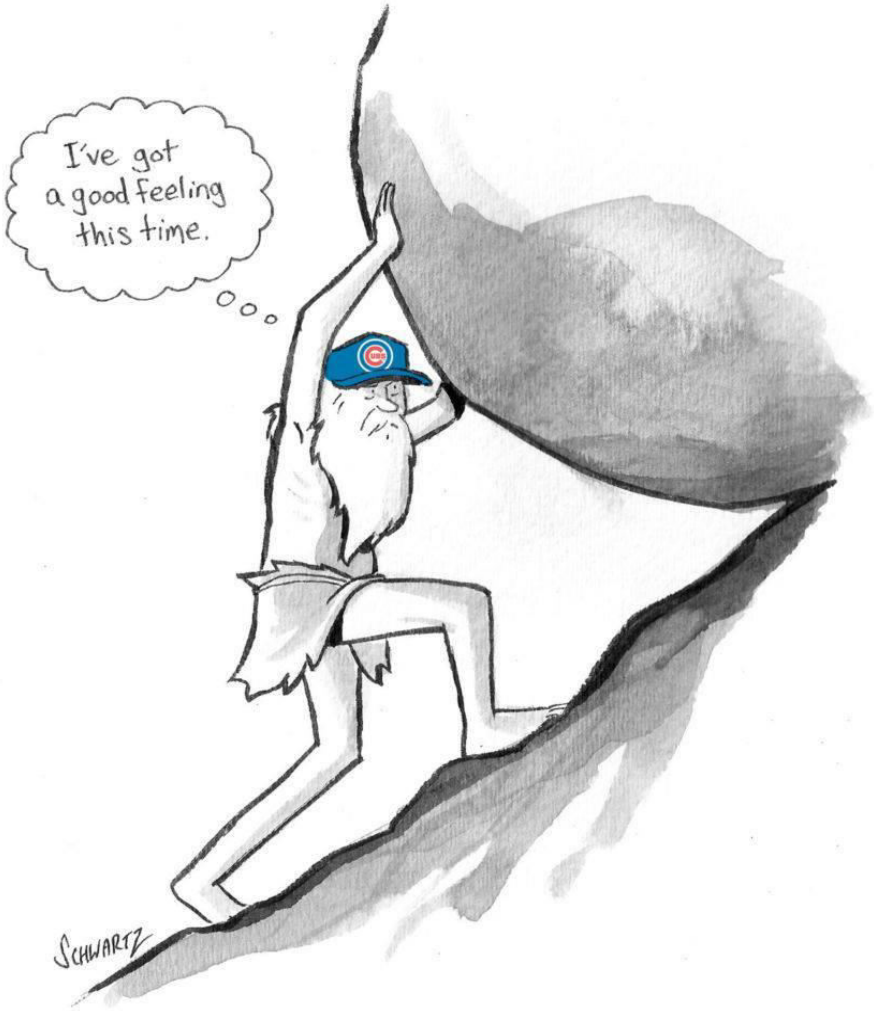


Figure S1. Uncropped western blots shown in figure 4. Full size western blots showing the expression of phosphorylated p38 (**a**), SAPK/JNK (**b**), and ERK1/2 (**c**). Membranes were probed with GAPDH to confirm equal loading. Horizontal lines mark the cropped sections of the blots shown in figure 4. A normalisation sample (NS) was used in each gel. Contrast settings were not modified in any image.

Chapter 4

Conclusions and outlook



Drawing from Azure, 7 November 2016.

The earlier PTB happens, the greater the health risks for the newborn baby. Despite considerable efforts in the past years, there is still an urgent need for more reliable treatments. It is of vast importance to find selective, well-tolerated and safe uteromodulating compounds to prevent complications before birth [1]. Nature is a rich source for the discovery of medical compounds which might help. Nevertheless, it is a challenge to find a suitable plant and characterise the compounds responsible for its beneficial effects. *B. pinnatum* is a very useful plant for treating various disorders and a well-regarded phytotherapeutic with potential to treat PTL [2, 3].

4.1 Closing remarks

In this thesis, we have shown over and again the importance of *B. pinnatum* for the contractility of the uterus.

More specifically, our results revealed that:

I. Press juice from ***B. pinnatum*** **leaves enhances** the inhibitory effect of the OTR antagonist **atosiban** and of **nifedipine**, a voltage-dependent calcium channel blocker, on contraction strength in human myometrium strips
II. **Bufadienolides** may be mainly responsible for the relaxant effect of *B. pinnatum* on myometrium strips contractility
III. *B. pinnatum* compounds are **not cytotoxic** even at concentrations higher than those needed for the effects on contractility
IV. *B. pinnatum* press juice promotes a **strong decrease** of the OT-induced increase of **$[Ca^{2+}]_i$** in two different myometrium cell lines and the **combination** of fractions enriched in **bufadienolides and in flavonoids** is necessary to obtain a similar inhibition
V. The combination of fractions enriched in **bufadienolides and in flavonoids** promotes **synergistic effect** on the OT-induced increase of $[Ca^{2+}]_i$
VI. *B. pinnatum* press juice inhibits OT-induced activation of the MAPKs **SAPK/JNK** and **ERK1/2** by **phosphorylation**
VII. **Bufadienolides** seem to be particularly relevant for the inhibitory effects of *B. pinnatum* on **SAPK/JNK** and **ERK1/2 activation**

Multiple setups including *in vitro* cultures and human myometrial tissue were used to gain insights on the inhibitory effect of *B. pinnatum* on myometrium contractility. The promising results presented will engender a series of follow-up studies on multiple research fronts.

4.2 General discussion

The use of tocolytic drugs is the main option for the treatment of PTL. Unfortunately, their use has been frequently associated with low efficacy in long-term treatment and adverse reactions [4-6]. Therefore, new drugs or herbal extracts are being tested in PTB models both in clinical studies and in *in vitro* models to explore their possible therapeutic benefits (for examples see [7, 8]). Various preparations of *B. pinnatum* are currently being used in a broad range of therapeutic indications [2, 9] and, in Switzerland, *B. pinnatum* has found wide use in tocolysis as an add-on therapy [10, 11]. This herbaceous plant allows a longer treatment, since some preparations can be taken orally, and has less side effects than known tocolytics [8, 12-15].

Synthesis

The first retrospective clinical trials documented a potent tocolytic effect of *B. pinnatum* [12-14], which led to experimental investigations using various *in vitro* models. Initial results of *in vitro* experiments using myometrial strips demonstrated a dose-dependent inhibitory effect of an aqueous leaf extract on spontaneous human myometrial contractions [16, 17], as well as an inhibition of OT-stimulated contractions [17]. Subsequent *in vitro* work showed that BPJ inhibits the OT-induced increase of $[Ca^{2+}]_i$ in myometrium cells and delays the depolarization-induced increase of $[Ca^{2+}]_i$ in cells with voltage-gated channels [18]. Taken together, these observations inspired us to investigate the effect of BPJ in combination with atosiban, an OTR antagonist, and nifedipine, a voltage-gated calcium channel blocker, on human myometrial contractility *in vitro*. The leaf press juice increased the inhibitory effect of atosiban and nifedipine, providing evidence for the add-on use of *B. pinnatum* during tocolysis.

Since *B. pinnatum* demonstrated efficient myometrium relaxant activities, the effect of the main metabolites, bufadienolides and flavonoids, was studied in a next step. Enriched fractions prepared from *B. pinnatum* leaves (BEF and FEF) led to a concentration-dependent decrease of

human myometrial contractions strength *in vitro*. Our results suggest that both BEF and FEF contain compounds that contribute to the effect of BPJ, but only BEF had an effect as strong as that of BPJ (at corresponding concentrations). Therefore, special attention should be paid to bufadienolides, since they seem to be the main responsible for the inhibitory effect of *B. pinnatum* on myometrium contractility.

The beginning of parturition is clinically manifested by rhythmic uterine contractions leading to the expulsion of the baby [19]. At cellular level, OT is a potent physiological stimulator of myometrial contractions, and its receptor and the subsequent intracellular mechanisms are attractive targets for drug development aimed at managing PTL [19, 20]. As mentioned before, previous *in vitro* work showed that BPJ inhibits the mobilisation of intracellular Ca^{2+} storages in myometrium cells (hTERT-C3) in a concentration-dependent way and, therefore, inhibits the canonical pathway of myometrial contractility [18]. In the present work, these observations were reproducible and comparable results were obtained with other myometrial cells (PHM1-41), showing that this effect is not restricted to one cell line. Furthermore, *B. pinnatum* fractions/compounds also promoted a concentration-dependent effect on the OT-induced increase of $[Ca^{2+}]_i$ in hTERT cells. However, their effects were not as strong as BPJ alone. Most interestingly, a strong and synergistic effect of fractions enriched in bufadienolides and in flavonoids could be observed (CI<1), proving that several compound classes in *B. pinnatum* contribute to the inhibition of the OT-induced increase of $[Ca^{2+}]_i$. Such a strong synergistic effect suggests that preparations of *B. pinnatum* leaves offer advantages over isolated compounds. This is in line with the opinion of other authors that positive interactions between compounds are likely to occur more frequently in traditionally used herbal preparations than is known [21, 22].

In the past years, the importance of inflammatory processes in labour at term and preterm have become apparent, and the MAPK pathway plays a central role in the activation of these processes [23]. It has been shown that $PGF_{2\alpha}$ is significantly released after stimulation with OT in pregnant and non-pregnant uteri suggesting a strong interaction between OT and PG in synergising the force of uterine contraction [24]. The investigation on the effect of BPJ compounds on the OT-induced activation of MAPKs show that BPJ, and in particular the corresponding bufadienolides, prevent the OT-induced phosphorylation of SAPK/JNK and

ERK1/2. This suggests that downstream enzymes involved in PG production might be inhibited, meaning that *B. pinnatum* would inhibit the inflammatory processes.

The setup

In vitro models can yield useful preliminary indications when the contractility of uterus is studied, but ultimately only *in vivo* experiments would provide a complete picture for a given tissue model. The use of complex animal models presents, however, an interspecies challenge for translation into humans, since the procedure of parturition is very specific in different animal species, and human parturition is not identical to that of other mammals [25, 26]. Several methods have been used to study myometrial contractility, from the generation of transformed or immortalized cells of myometrial origin, to *in vivo* internal and external tocography and intrauterine pressure catheters [27-29]. During this work, we took advantage of the following two different methods that allowed us to use human myometrium smooth muscle materials.

Immortalized cell lines combine properties of primary cells and the long life of continuous cell lines. These cells retain many morphological and phenotypic characteristics and are useful for long-term or coordinated studies, as their behaviour is quite reproducible. Still, like any *in vitro* model, the use of immortalised cells has its limitations, since the immortalisation of the cells can lead to different expression profiles. In the case of hTERT-C3, the cell line most often used in the present dissertation, a previous study has shown similar high expression levels of OTR as in primary myometrial cells corroborating the use of these immortalised cells as an *in vitro* model for the study of myometrium contractility [30].

Whilst cell culture systems can detect whether and how a substance acts at the cellular level, strips of tissues can be used as tools to measure functional responses of whole tissues to pharmacological agents. Strips of myometrium have been used by a number of research groups to examine many questions relating to myometrium physiology and pathology, including preterm [7, 31-33]. This technique allows the assessment of tissue contraction performance and the study of direct effects of different substances, alone or in combination, on contractility parameters. As the isolated tissue strips constitutes a model of more than one cell type, the physiological response of the whole tissue can be measured. Therefore, and in particular when

working with human material, the organ bath and isolated tissue strips are a useful tool in providing the bridge between cell culture work and whole animal/clinical studies. In the field of drug discovery, the effects of novel agents in terms of their excitatory or inhibitory potential for contractility *in vivo* can be more closely assessed. For example, the use of this method helped to validate the translational value of atosiban and generated the proof-of-concept data needed to take it forward to clinical trials [34-37].

Basic science vs clinical translation – bench to bedside

Despite the efforts in choosing the right assays to characterise the effect of *B. pinnatum* on uterus contractility, clinical translation remains the biggest challenge in the field. Knowledge pitfalls in some aspects of the use of *B. pinnatum* for the treatment of PTL remains a challenge for tocolysis in clinical practice. Thus, new approaches may lead to better clinical translation.

Nevertheless, in the course of this work we obtained promising data that can help understanding how *B. pinnatum* might affect the contractility of myometrium, but also may contribute to obtain better clinical readouts. Our results suggest that:

I. ***B. pinnatum* combined with atosiban or nifedipine might allow the lowering of required dosages of these well-known tocolytics**. These combinations would allow lowering medication costs and reducing maternal and foetus side-effects, allowing an increase of the time delay of pregnancy;
II. **Bufadienolides are important, but *B. pinnatum* is needed as a whole**. When we compare the effects of fractions, their combinations and BPJ, we can conclude that more than one compound is needed to obtain stronger effects. Developing a single-compound preparation might have limited effectiveness;
III. ***B. pinnatum* can also help in preventing PTL through a decrease in pro-inflammatory factors**. *In vitro* work with myometrium cells showed that *B. pinnatum* decreases the intensity of myometrial contractility through inhibition of several OT-induced signalling pathways. One of the pathways affected was the MAPK-pathway, which leads to PG production and further activation of contractility through inflammation.

Furthermore, the following limitations might impair a better translation of results: i) there is a limited correspondence between concentrations used in *in vitro* studies and attainable in

patients, since no pharmacokinetic data on *B. pinnatum* (compounds) are yet available, not even during pregnancy; and ii) possible metabolisation of *B. pinnatum* is not being taken into consideration. These limitations are further discussed in the following subchapter.

4.3 Future directions

We believe that the results obtained during this project are a stepping stone for future research. The phytotherapy research field is rapidly evolving and important consideration must be undertaken for future studies in order to unify findings and be able to build on each others' results. During the course of this work, we came across some questions related to BPJ concentrations which limited the translation of our results to the clinics. These limitations were mentioned previously in "Basic science vs clinical translation – bench to bedside". The first limitation could be overcome by performing pharmacokinetic studies with *B. pinnatum* compounds. Interestingly, the second limitation is related to the first, and could be solved by studying what happens to *B. pinnatum* from the moment it is taken until it reaches the blood stream. The preparation from *B. pinnatum* press juice most commonly used in Switzerland, Bryophyllum 50% chewable tablets, are taken orally, and thus both intestinal and liver metabolisation need to be considered. Such information would be useful in the planning of future prospective randomised studies that are urgently needed to definitely prove the efficacy of *B. pinnatum* preparations, alone or in combination with standard tocolytics.

In the future, it would also be useful to deepen knowledge on the OT-induced pathways affected by *B. pinnatum*. Regulation of upstream or downstream proteins, gene expression and, especially PG production deserves further investigation. Since in this work we showed that *B. pinnatum* affects MAPK activation, it is likely that it affects PG production. Thus, in future studies, the effect of *B. pinnatum* on myometrium contractility should also be evaluated from the perspective of inflammation.

Clinical practice shows that not all patients benefit equally from treatment with agents acting on OTR, meaning that patients' response to these medications is highly variable. The existence of genetically variant forms of OTR are likely to influence patient responses to the medications that bind to this receptor. Our previous work showed that genetic variants of the *OTR gene*

might have an impact on myometrial contractility strength induced by OT [38]. Since the results described in Chapter 3 strongly suggest that *B. pinnatum* compounds act on OTR, they also pose some new questions: i) Do genetic variant forms of *OTR gene* also affect the impact of *B. pinnatum* on human myometrium contractility? and ii) Which variant forms are more likely to have an impact on the effect of *B. pinnatum*? To answer these research questions, additional work will be needed.

4.4 References

1. Arrowsmith S, Kendrick A, and Wray S (2010). Drugs acting on the pregnant uterus. *Obstet Gynaecol Reprod Med*. 20(8): 241-247.
2. Hamburger M, Potterat O, Furer K, Simoes-Wüst AP, and von Mandach U (2017). *Bryophyllum pinnatum*-Reverse Engineering of an Anthroposophic Herbal Medicine. *Nat Prod Commun*. 12(8): 1359-1364.
3. Fürer K, Simões-Wüst AP, von Mandach U, Hamburger M, and Potterat O (2016). *Bryophyllum pinnatum* and related species used in anthroposophic medicine: constituents, pharmacological activities, and clinical efficacy. *Planta Medica*. 82(11/12): 930-941.
4. Hubinont C, and Debiève F (2011). Prevention of preterm labour: 2011 update on tocolysis. *J Pregnancy*.
5. Hösli I, Sperschneider C, Drack G, Zimmermann R, Surbek D, and Irion O (2014). Tocolysis for preterm labor: Expert opinion. *Arch Gynecol Obstet*. 289(4): 903-909.
6. ACOG: American College of Obstetricians and Ginecologists (2016). Practice Bulletin No. 171: Management of Preterm Labor. *Obstet Gynecol*. 128(4): e155.
7. Kim SH, Riaposova L, Ahmed H, Pohl O, Chollet A, Gotteland JP, Hanyaloglu A, Bennett PR, and Terzidou V (2019). Oxytocin receptor antagonists, atosiban and nolasiban, inhibit prostaglandin F2alpha-induced contractions and inflammatory responses in human myometrium. *Sci Rep*. 9(1): 5792.
8. Simões-Wüst AP, Lapaire O, Hösli I, Wächter R, Fürer K, Schnelle M, Mennet-von Eiff M, Seifert B, and von Mandach U (2018). Two randomised clinical trials on the use of *Bryophyllum pinnatum* in pre-term labour – results after discontinuation. *Complement Med Res*.
9. Simões-Wüst AP, Jeschke E, Mennet M, Schnelle M, Matthes H, and von Mandach U (2012). Prescribing pattern of *Bryophyllum* preparations among a network of anthroposophic physicians. *Complement Med Res*. 19(6): 293-301.
10. Fürer K, Simões-Wüst AP, Winkler A, Amsler N, Schnelle M, and von Mandach U (2015). Die Anwendung von *Bryophyllum pinnatum* - Präparaten in der Geburtshilfe und Gynäkologie-eine multizentrische prospektive Beobachtungsstudie. *Complement Med Res*. 22(4): 231-236.
11. Schenkel L, Simões-Wüst AP, Hoesli I, and von Mandach U (2018). Drugs in pregnancy and lactation - medications used in Swiss obstetrics. *Z Geburtsh Neonatol*. 222: 1-14.
12. Hassauer W, Schreiber K, and Von der Decken D (1985). Ein neuer Weg in der tokolytischen Therapie. *Erfahrungsheikunde*. 34: 683-687.
13. Daub E, *Vorzeitige Wehentätigkeit: ihre Behandlung mit pflanzlichen Substanzen: eine klinische Studie*. 1989: Urachhaus.
14. Vilàghy (2002). Decrease in the incidence of premature deliveries with the use of phytotherapy – results of clinical practice. *Therapeutische Umschau*. 59(12): 696-701.

15. Plangger N, Rist L, Zimmermann R, and von Mandach U (2006). Intravenous tocolysis with *Bryophyllum pinnatum* is better tolerated than beta-agonist application. *Eur J Obstet Gynecol Reprod Biol*. 124(2): 168-172.

16. Wächter R, Brenneisen R, Hamburger M, Mennet M, Schnelle M, Worel A, Simões-Wüst AP, and Von Mandach U (2011). Leaf press juice from *Bryophyllum pinnatum* (Lamarck) Oken induces myometrial relaxation. *Phytomedicine*. 19(1): 74-82.

17. Gwehenberger B, Rist L, Huch R, and von Mandach U (2004). Effect of *Bryophyllum pinnatum* versus fenoterol on uterine contractility. *Eur J Obstet Gynecol Reprod Biol*. 113(2): 164-171.

18. Simões-Wüst AP, Grãos M, Duarte C, Brenneisen R, Hamburger M, Mennet M, Ramos M, Schnelle M, Wächter R, and Worel A (2010). Juice of *Bryophyllum pinnatum* (Lam.) inhibits oxytocin-induced increase of the intracellular calcium concentration in human myometrial cells. *Phytomedicine*. 17(12): 980-986.

19. Aguilar HN, and Mitchell B (2010). Physiological pathways and molecular mechanisms regulating uterine contractility. *Human Reprod Update*. 16(6): 725-744.

20. Gulliver D, Werry E, Reekie TA, Katte TA, Jorgensen W, and Kassiou M (2018). Targeting the oxytocin system: new pharmacotherapeutic approaches. *Trends Pharmacol Sci*. 40(1): 22-37.

21. Butterweck V, and Nahrstedt A (2012). What is the best strategy for preclinical testing of botanicals? A critical perspective. *Planta Medica*. 78(08): 747-754.

22. Wagner H (2011). Synergy research: approaching a new generation of phytopharmaceuticals. *Fitoterapia*. 82(1): 34-37.

23. Kim SH, Pohl O, Chollet A, Gotteland J-P, Fairhurst AD, Bennett PR, and Terzidou V (2017). Differential effects of oxytocin receptor antagonists, Atosiban and Nolasiban, on OT-mediated signalling in human amnion and myometrium. *Mol Pharmacol*. 91(4): 403-415.

24. Alotaibi MF (2017). The response of rat and human uterus to oxytocin from different gestational stages in vitro. *Gen Physiol Biophys*. 36(1): 75-82.

25. Muglia LJ, and Katz M (2010). The enigma of spontaneous preterm birth. *N Engl J Med*. 362(6): 529-535.

26. Smith R (2007). Parturition. *N Engl J Med*. 356(3): 271-283.

27. Condon J, Yin S, Mayhew B, Word RA, Wright W, Shay J, and Rainey WE (2002). Telomerase immortalization of human myometrial cells. *Biol Reprod*. 67(2): 506-514.

28. Newman RB (2005). Uterine contraction assessment. *Obstetrics and Gynecology Clinics*. 32(3): 341-367.

29. Haran G, Elbaz M, Fejgin MD, and Biron-Shental T (2012). A comparison of surface acquired uterine electromyography and intrauterine pressure catheter to assess uterine activity. *AJOG: Am J Obstet Gynecol*. 206(5): 412. e1-412. e5.

30. Devost D, and Zingg HH (2007). Novel in vitro system for functional assessment of oxytocin action. *Am J Physiol Endocrinol Metab*. 292(1): E1-E6.

31. Arulkumaran S, Kandola MK, Hoffman B, Hanyaloglu AC, Johnson MR, and Bennett PR (2012). The roles of prostaglandin EP 1 and 3 receptors in the control of human myometrial contractility. *J Clin Endocrinol Metab*. 97(2): 489-498.

32. David M, Hamann C, Chen FC-K, Bruch L, and Lichtenegger W (2000). Comparison of the relaxation effect *in vitro* of nitroglycerin vs. fenoterol on human myometrial strips. *J Perinat Med*. 28(3): 232-242.

33. Stoiber B, Haslinger C, Schäffer M, Zimmermann R, and Schäffer L (2018). Effect of dual tocolysis with fenoterol and atosiban in human myometrium. *J Perinat Med*.

34. Goodwin TM, Millar L, North L, Abrams LS, Weglein RC, and Holland ML (1995). The pharmacokinetics of the oxytocin antagonist atosiban in pregnant women with preterm uterine contractions. *AJOG: Am J Obstet Gynecol*. 173(3): 913-917.

35. Goodwin TM, Paul R, Silver H, Spellacy W, Parsons M, Chez R, Hayashi R, Valenzuela G, Creasy GW, and Merriman R (1994). The effect of the oxytocin antagonist atosiban on preterm uterine activity in the human. *AJOG: Am J Obstet Gynecol*. 170(2): 474-478.

36. Goodwin TM, Valenzuela G, Silver H, Hayashi R, Creasy GW, and Lane R (1996). Treatment of preterm labor with the oxytocin antagonist atosiban. *Am J Perinatol*. 13(03): 143-146.

37. Romero R, Sibai BM, Sanchez-Ramos L, Valenzuela GJ, Veille J-C, Tabor B, Perry KG, Varner M, Goodwin TM, and Lane R (2000). An oxytocin receptor antagonist (atosiban) in the treatment of preterm labor: a randomized, double-blind, placebo-controlled trial with tocolytic rescue. *AJOG: Am J Obstet Gynecol*. 182(5): 1173-1183.

38. Füeg F, Santos S, Haslinger C, Stoiber B, Schäffer L, Grünblatt E, Zimmermann R, and Simões-Wüst AP (2019). Influence of oxytocin receptor single nucleotide sequence variants on contractility of human myometrium: an in vitro functional study. *BMC Med Genetics.* 20(1): p. 178.