Treatment of mild mental disorders in pregnancy: How safe are phytomedicines?

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Erstbetreuerin	PD Dr. Ana Paula Simões-Wüst
Zweitbetreuer	Prof. Dr. Matthias Hamburger
Externer Experte	Prof. Dr. Udo Markert

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Prof. Dr. Marcel Mayor
Dekan

To my beloved mother

“I have never tried that before,

so I think I should definitely be able to do that”

Astrid Lindgren

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SUMMARY

Pregnancy and associated physiological changes, which begin in the first trimester and are most pronounced in the third trimester, alter the pharmacokinetics of drugs. In addition, most drugs enter the fetal-placental unit, which pose several challenges to medical care, as it affects both the unborn child as well as the expectant mother. This also applies to the treatment of non-psychotic mental disorders (NMDs) such as depression, restlessness, sleep disorders, and anxiety since the use of synthetic, central nervous system (CNS)-active drugs for treatment should be carefully considered in a risk-benefit ratio. Nevertheless, pregnancy is a vulnerable period for such disorders and preexisting mental illnesses may even worsen or relapse. Alternatively, there are some herbal medicines, so called phytopharmaceuticals, which possess antidepressant, sedative, or anxiolytic properties. St. John's wort, for example, is used to treat mild to moderate depression, whereas California poppy, valerian, and hops are mainly known for their sleep-inducing effects. Finally, lavender essential oil represents an option for the treatment of restlessness and anxiety due to its calming, sedative, and anxiolytic effects. Most products based on these phytopharmaceuticals are available over the counter (OTC) and are generally recommended by a considerable proportion of health care professionals who deal with pregnant women, such as midwives and obstetricians. Despite the limited experimental and clinical evidence, and especially lack of safety data, pregnant women often resort to herbal medicines and perceive them as safe.

We designed a cross-sectional survey with which we determined the use of most common herbal medicines in pregnancy by obstetrics patients. Furthermore, some questions targeted the perceived effectiveness and tolerability of some of these plants. The survey revealed that a large majority of pregnant women make use of herbal medicines, and lavender (22%), valerian (4.7%), St. John's wort (3.0%), and hops (1.7%) were among the most mentioned pharmaceutical herbal products for the treatment of mental disorders and/or symptoms (MDS). Although 52.0% of all participants suffered from MDS during pregnancy, only 1.3% reported taking (synthetic) psychoactive medications. The fact that the prevalence of MDS was higher in pharmaceutical herbal products users than in non-users suggests that pregnant women rely on herbal medicines to treat mild MDS. St. John's wort, valerian, hops, and lavender were used to reduce mood and sleep disorders, restlessness, and stress with perceived good to very good effectiveness and tolerability; no participant reported the use of California poppy.

To verify whether various herbal extracts such as St. John's wort, California poppy, valerian, hops, and lavender can be used for the treatment of NMD in pregnancy, their safety must be thoroughly investigated with a palette of *in vitro* assays. Those extracts and some of their active marker compounds were therefore evaluated for their cytotoxic and genotoxic potential and for their effects on metabolism and cell differentiation. The in-depth *in vitro* safety assessment indicates that extracts of St. John's wort, California poppy, valerian, hops, and lavender – at concentrations up to 30 μg/mL – have no cytotoxic or genotoxic potential and do not compromise the viability, metabolic activity, and differentiation of placental cells. Moreover, none of the five extracts was able to significantly alter protein expression of BeWo b30 cells. For active marker substances, protopine (found in California poppy), valerenic acid (in valerian), and linalool (in lavender) showed no adverse effects in all experiments performed. The following phytochemicals might conceivably cause safety issues: hyperforin (in St. John's wort) induced cell apoptosis (≥ 3 μM) and inhibited BeWo b30 cell differentiation (≥ 1 μM). Hypericin (in St. John's wort) decreased cell viability (≥ 1 μM) and induced cell apoptosis (30 μM). Valtrate (in valerian) decreased cell viability (≥ 10 μM), induced cell apoptosis (≥ 10 μM), and decreased the metabolic cell activity by reducing glucose consumption and concomitant lactate production. However, none of the tested phytochemicals resulted in genotoxic effects and thus are not DNA damaging. In summary, most of the phytochemicals were not of concern, but the attainment of high plasma concentrations of a few relevant phytochemicals – hyperforin, hypericin, and valtrate – deserves special attention.

In addition to investigating effects on placental cells, the question of transplacental permeation of certain phytochemicals needs to be addressed in order to better assess the risk of fetal exposure. For this purpose, an *ex vivo* placental perfusion model was optimized and modified that it can be used for the application of phytochemicals. Furthermore, an *in vitro* Transwell model with placental cells was established which served to complement the placental perfusion model. We successfully used the human *ex vivo* placental perfusion model for the first time for transport experiments with phytochemicals, after a thorough validation with various compounds, such as antipyrine, citalopram, and diazepam.

Only a small fraction of the initially present hyperforin (< 10%) reached the fetal circuit after 4 h, whereas hypericin did not cross the placental barrier and therefore remained in the maternal circuit. In contrast, protopine was transferred from the maternal to the fetal circuit, reaching a steady state after 90 min with no further changes in concentration. Also, valerenic acid was transferred gradually over a 4-hour period and reached an equilibrium with the maternal concentration. None of the phytochemicals affected placental viability or functionality, and histopathological evaluation of all placental specimens revealed no pathologic findings. In addition,

in vitro translocation studies have confirmed that valerenic acid, from valerian, cannot cross the placental cell layer within 60 min, which might indicate that this phytochemical is not accessible to the unborn child in early pregnancy.

We were able to gain more detailed knowledge into the safety of St. John's wort, California poppy, valerian, hops, and lavender extracts and a few of their active phytochemicals through in-depth *in vitro* assessments and an *ex vivo* model. In addition, we know that phytomedicines of these extracts, except California poopy, are already used in pregnancy in Switzerland with well-perceived effectiveness and tolerability. We hope that this thesis contributes to a rational basis for future decisions on the treatment of NMDs during pregnancy.

ZUSAMMENFASSUNG

Die Schwangerschaft und die damit verbundenen physiologischen Veränderungen, die im ersten Trimester beginnen und im dritten Trimester am stärksten ausgeprägt sind, verändern die Pharmakokinetik von Arzneimitteln. Darüber hinaus gelangen die meisten Arzneimittel in die fetoplazentare Einheit. Dies stellt die medizinische Versorgung vor einige Herausforderungen, da neben dem Wohl der werdenden Mutter auch die Auswirkungen auf das ungeborene Kind berücksichtigt werden müssen. Dies gilt auch für die Behandlung nicht-psychotischer psychischer Erkrankungen wie Depressionen, Unruhezustände, Schlafstörungen und Angstzustände, da der Einsatz synthetischer, ZNS-aktiver Medikamente zur Behandlung in einem Risiko-Nutzen-Verhältnis sorgfältig abgewogen werden sollte. Dennoch ist die Schwangerschaft ein gefährdeter Zeitraum für die Entstehung solcher Erkrankungen, wobei sich bereits bestehende psychische Erkrankungen sogar verschlimmern können. Alternativ gibt es einige pflanzliche Präparate (sog. Phytopharmaka), die antidepressive, sedierende oder anxiolytische Eigenschaften besitzen. Johanniskraut beispielsweise wird zur Behandlung leichter bis mittelschwerer Depressionen eingesetzt, während Extrakte aus kalifornischem Mohn, Baldrian und Hopfen vor allem für ihre schlaffördernde Wirkung bekannt sind. Das ätherische Lavendelöl ist aufgrund seiner beruhigenden, sedierenden und angstlösenden Wirkung eine Option für die Behandlung von Unruhe und Angstzuständen. Die meisten Phytopharmaka sind rezeptfrei erhältlich und werden im Allgemeinen von einem beträchtlichen Teil der medizinischen Fachkräfte (v.a. Hebammen und Geburtshelfern), die mit schwangeren Frauen interagieren, empfohlen. Trotz der begrenzten experimentellen und klinischen Nachweise und insbesondere des Mangels an Sicherheitsdaten greifen schwangere Frauen häufig auf pflanzliche Präparate zurück und halten sie für sicher.

Wir entwarfen eine Querschnittserhebung, mit der wir die Verwendung der gängigsten pflanzlichen Zubereitungen in der Schwangerschaft durch Patientinnen der Geburtshilfe ermittelten. Außerdem zielten einige dieser Fragen auf die wahrgenommene Wirksamkeit und Verträglichkeit einiger dieser Pflanzen ab. Die Umfrage ergab, dass eine große Mehrheit der schwangeren Frauen pflanzliche Arzneimittel verwendet, wobei Lavendel (22.0%), Baldrian (4.7%), Johanniskraut (3.0%) und Hopfen (1.7%) zu den am häufigsten genannten pflanzlichen Arzneimitteln für die Behandlung von nicht-psychotischer psychischer Erkrankungen gehörten. Obwohl 52.0% aller Teilnehmer-innen während der Schwangerschaft an solchen Erkrankungen litten, gaben nur 1.3% an, (synthetische) psychoaktive Medikamente einzunehmen. Die Tatsache, dass die Prävalenz von psychischen Störungen und/oder Symptomen bei Anwenderinnen von pflanzlichen Arzneimitteln höher war als bei Nichtanwenderinnen, deutet darauf hin, dass schwangere Frauen bei deren

Behandlung auf pflanzliche Arzneimittel zurückgreifen. Johanniskraut, Baldrian, Hopfen und Lavendel wurden zur Verringerung von Stimmungs- und Schlafstörungen, Unruhe und Stress eingesetzt, wobei die Wirksamkeit und Verträglichkeit teilweise als gut bis sehr gut eingeschätzt wurde. Keine Teilnehmende berichtete über die Verwendung von kalifornischem Mohn während der Schwangerschaft.

Um zu überprüfen, ob verschiedene pflanzliche Extrakte wie Johanniskraut, kalifornischer Mohn, Baldrian, Hopfen und Lavendel für die Behandlung von nicht-psychotischer psychischer Erkrankungen in der Schwangerschaft verwendet werden können, muss ihre Sicherheit mit einer Reihe von i*n vitro* Assays gründlich untersucht werden. Diese Extrakte und einige ihrer aktiven Hauptverbindungen wurden daher auf ihr zytotoxisches und genotoxisches Potenzial sowie auf ihre Auswirkungen auf den Stoffwechsel und die Zelldifferenzierung untersucht. Die detaillierte *in vitro* Sicherheitsuntersuchung zeigt, dass Extrakte aus Johanniskraut, kalifornischem Mohn, Baldrian, Hopfen und Lavendel – in Konzentrationen von bis zu 30 μg/mL – kein zytotoxisches oder genotoxisches Potenzial haben und die Lebensfähigkeit, Stoffwechselaktivität und Differenzierung von Plazentazellen nicht beeinträchtigen. Außerdem war keiner der fünf Extrakte in der Lage, die Proteinexpression der BeWo b30 Zellen signifikant zu verändern. Was die aktiven Hauptinhaltsstoffe betrifft, so zeigten Protopin (in kalifornischem Mohn enthalten), Valerensäure (in Baldrian) und Linalool (in Lavendel) in allen durchgeführten Experimenten keine nachteiligen Auswirkungen. Die folgenden Phytochemikalien könnten möglicherweise Sicherheitsprobleme verursachen: Hyperforin (in Johanniskraut) löste Zellapoptose aus (≥ 3 μM) und hemmte die Differenzierung von BeWo b30 Zellen (≥ 1 μM). Hypericin (in Johanniskraut) verringerte die Lebensfähigkeit der Zellen (≥ 1 μM) und induzierte die Apoptose der Zellen (30 μM). Valtrat (in Baldrian) verringerte die Lebensfähigkeit der Zellen (≥ 10 μM), induzierte die Zellapoptose (≥ 10 μM) und verringerte die metabolische Zellaktivität durch Verringerung des Glukoseverbrauchs und gleichzeitigen Laktatproduktion. Keine der getesteten Inhaltsstoffe führte jedoch zu genotoxischen Wirkungen und sind somit nicht DNA-schädigend. Zusammenfassend lässt sich sagen, dass die meisten Phytochemikalien nicht besorgniserregend waren, aber das Erreichen hoher Plasmakonzentrationen einiger relevanter Phytochemikalien – Hyperforin, Hypericin und Valtrat – verdient besondere Aufmerksamkeit.

Neben den Auswirkungen von Extrakten und Phytochemikalien auf Plazentazellen muss auch die Frage der transplazentaren Permeation bestimmter Phytochemikalien geklärt werden, um das Risiko einer fetalen Exposition besser einschätzen zu können. Zu diesem Zweck wurde ein *ex vivo* Plazenta-Perfusionsmodell optimiert und so modifiziert, dass es für die Anwendung von Phytochemikalien verwendet werden kann. Außerdem wurde ein *in vitro* Transwell-Modell mit Plazentazellen etabliert, das als Ergänzung zum Plazenta-Perfusionsmodell dient. Wir haben mit dem humanen *ex vivo* Plazenta-Perfusionsmodell zum ersten Mal erfolgreich Transportexperimente mit Phytochemikalien durchgeführt, nachdem wir mit verschiedenen Verbindungen wie Antipyrin, Citalopram und Diazepam eine gründliche Validierung durchgeführt hatten.

Nur ein kleiner Teil des ursprünglich vorhandenen Hyperforins (< 10 %) erreichte nach 4 Stunden den fetalen Kreislauf, während Hypericin die Plazentaschranke nicht passieren konnte und daher im mütterlichen Kreislauf verblieb. Protopin wurde vom mütterlichen in den fetalen Kreislauf transferiert und erreichte nach 90 Minuten einen Steady-State ohne weitere Konzentrationsänderungen. Im Gegensatz dazu wurde Valerensäure über einen Zeitraum von 4 Stunden schrittweise übertragen und erreichte dann mit der mütterlichen Konzentration ein Gleichgewicht. Keine der getesteten Phytochemikalien beeinträchtigte die Lebensfähigkeit oder Funktionalität der Plazenta. Auch die histopathologische Auswertung aller Plazentaproben ergab keine pathologischen Befunde. Darüber hinaus haben *in vitro* Transportstudien bestätigt, dass Valerensäure (in Baldrian) die Zellschicht der Plazenta innerhalb von 60 Minuten nicht durchqueren kann, was darauf hindeutet, dass Valerensäure in der Frühschwangerschaft für das ungeborene Kind nicht zugänglich ist.

Mithilfe eingehender *in vitro* Untersuchungen und Gebrauch eines *ex vivo* Modells konnten wir genauere Erkenntnisse über die Sicherheit von Extrakten aus Johanniskraut, kalifornischem Mohn, Baldrian, Hopfen und Lavendel sowie einiger ihrer aktiven Inhaltsstoffe gewinnen. Darüber hinaus wissen wir, dass pflanzliche Zubereitungen dieser Extrakte in der Schweiz in der Schwangerschaft bereits eingesetzt werden und sich als wirksam und gut verträglich erwiesen haben. Wir hoffen, dass diese Arbeit eine rationale Grundlage für zukünftige Entscheidungen über die Behandlung von nicht-psychotischer psychischer Erkrankungen in der Schwangerschaft liefert.

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I dedicate this work to my *mother*. There is probably no greater role model than her when it comes to perseverance and strength. I am very grateful that you never gave up and I am very proud of you.

LIST OF ABBREVIATIONS

AD	Anno Domini (= in the year of the Lord)
BCE	Before the Common Era
BCRP	Breast cancer resistance protein
CAM	Complementary and alternative medicine
CNS	Central nervous system
CREB	cAMP response element-binding protein
CRH	Corticotropin-releasing hormone
CYP	Cytochrome P450
DSM-5	Diagnostic and Statistical Manual of Mental Disorders
EEG	Electroencephalogram
EGF	Epidermal growth factor
EMA	European Medicines Agency
EPR	Electronic Patient Record
ESCOP	European Scientific Cooperative on Phytotherapy
EU	European Union
FGCZ	Functional Genomics Center Zurich
GABA	γ-aminobutyric acid
GC/MS	Gas Chromatography/Mass Spectrometry
GDM	Gestational diabetes mellitus
GnRH	Gonadotropin-releasing hormone
HMPC	Committee for Herbal Medicinal Products
HPEC	Pure vascular endothelial cell line from the human placenta
hPL	Human placental lactogen
HUVECs	Human umbilical vein endothelial cells
ICD-10	International Classification of Diseases
IGF	Insulin-like growth factors
IgG	Immunoglobulin G
IUGR	Intrauterine growth restriction
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
MAPK	Mitogen-activated protein kinase
MATE1	Multidrug and toxin extruding protein 1

MDR1	Multidrug resistance protein 1
MRP1	Multidrug resistance-associated protein 1
MRP2	Multidrug resistance-associated protein 2
NAS	Neonatal adaptation syndrome
NMDs	Non-psychotic mental disorders
OAT4	Organic anion transporter 4
OATP2B1	Organic anion transporting polypeptide 2B1
OATP4A1	Organic anion transporting polypeptide 4A1
OCT3	Organic cation transporter 3
OCTN1	Novel organic cation transporter 1
OCTN2	Novel organic cation transporter 2
OTC	Over the counter
P-gp	P-glycoprotein
PBPK	Physiologically based pharmacokinetic
PDGF	Platelet-derived growth factor
PGH	Placental growth hormone
Ph. Eur.	European Pharmacopoeia
PKA	Protein kinase A
QSAR	Quantitative structure-activity relationship
SAPP	Schweizerische Akademie für perinatale Pharmakologie
SNRI	Serotonin-norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
TDM	Therapeutic drug monitoring
TRPC 6	Transient receptor potential channel 6
UGT	UDP-glucuronosyltransferase
USA	United States of America
VDCC	Voltage dependent calcium channels
WHO	World Health Organization
β-hCG	Beta-human chorionic gonadotropin

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1 SCOPE OF THE THESIS

Women who need to treat health conditions during their pregnancy often face a major dilemma, as the pharmacological therapies could be potentially harmful to the unborn child. This is also true for the treatment of non-psychotic mental disorders such as depression, restlessness, sleep disorders, and anxiety, which is why various phytomedicines are available as potential alternatives. These show good clinical efficacy, however safety data on reproductive toxicology are only partially available or even completely lacking. It is becoming increasingly evident that a better understanding of the safety of herbal medicines in pregnancy is urgently needed. The main objective of this thesis was therefore to assess the safety of various herbal extracts and phytochemicals by studying their effects on placental function *in vitro* and *ex vivo*, and to investigate on the transplacental transfer of phytochemicals to evaluate the fetal exposure.

The specific aims of this thesis were:

(i) to reveal the most commonly used herbal medicines in pregnancy, in the presence of MDS, and to evaluate some of their perceived effectiveness and tolerability with a cross-sectional survey of obstetrics patients ***(Chapter 1)***.

(ii) to evaluate the safety of various herbal extracts (St. John's wort, California poppy, valerian, hops, lavender) in pregnancy, with an *in vitro* assessment of their cytotoxic and genotoxic potential in a placental cell line (BeWo b30). In addition, we aimed to characterize their impact on critical metabolic properties such as glucose consumption and lactate production, as well as their ability to induce or inhibit cell differentiation ***(Chapter 2)***.

(iii) to assess the influence of herbal extracts on the proteome of the placental cell line BeWo b30 and to establish a safety profile of some of the plant extracts active marker compounds such as hyperforin and hypericin (in St. John's wort), protopine (in California poppy), valerenic acid and valtrate (in valerian), and linalool (in lavender) using the same plethora of *in vitro* assays as for the extracts ***(Chapter 3)***.

(iv) to clarify the permeation of hyperforin, hypericin, protopine, and valerenic acid across the placental barrier to better estimate the risk of fetal exposure using the *ex vivo* human placental perfusion model, and to investigate the translocation of valerenic acid across BeWo b30 cells by establishing an *in vitro* Transwell model ***(Chapter 4 and 5)***.

2 INTRODUCTION

2.1 Perinatal Pharmacology

During pregnancy, various physiological changes take place in the body of a woman over the course of nine months, some of which can affect the pharmacokinetics and thus the efficacy of drugs in very different ways, inevitably leading to adjustments (e.g., dose) during pregnancy to make therapy as effective and safe as possible. All processes of pharmacokinetics – from absorption, distribution, and metabolism to excretion of a drug – can be affected [1-3] and are discussed below.

2.1.1 Physiological and pharmacokinetic changes

Absorption

The physiological changes that occur during pregnancy include changes in the gastrointestinal tract, such as prolonged gastric emptying time (> 6 hours) due to decreased intestinal motility and increased gastric pH, which affect drug absorption when delivered via the oral route. Absorption of inhaled drugs may be enhanced due to increased cardiac output and tidal volume, which increase alveolar uptake, resulting in decreased dose requirements in pregnancy. Absorption of intramuscular administered drugs is also usually improved because tissue perfusion is increased because of vasodilation.

Distribution

During pregnancy, body composition changes, which is mainly associated with the increase of intravascular (plasma volume) and extravascular (breasts, uterus, peripheral oedema) body water content. Overall, the total body water increases by up to 8 liters, increasing the distribution volume in which hydrophilic drugs may distribute. Furthermore, the plasma concentrations of albumin-bound drugs decrease, as the protein concentration in the blood decreases due to increased water reabsorption. The cardiovascular system is also more stressed during pregnancy, as blood volume, cardiac output, and heart rate increase. Lastly, the additional utero-fetal compartment as well as the increase in body fat by approximately 4 kg change the distribution volume of certain drugs. The latter mainly affects lipophilic substances.

Metabolism

Metabolism can be increased by elevated liver blood flow. Hormones such as estrogens and progesterone have an important influence on hepatic cytochrome P450 enzyme (CYP) activity, which induces or even completely inhibits some isoenzymes, leading to an altered metabolism and elimination. Furthermore, activity of phase II enzymes such as UDP-glucuronosyltransferases (UGTs) are increased in pregnancy.

Elimination

Renal blood flow is increased by 60-80% during pregnancy, resulting in increased glomerular filtration. Due to this, the pregnant woman often notices increased urination (= physiological polyuria). In addition, various transport proteins are induced in the kidney which further increase the active excretion of certain drugs. Although increased elimination results in slightly lower steady-state drug concentrations, dosages rarely need to be increased.

Dose adjustment is required for various drug classes, such as for psychotropic drugs. A dose increase may be appropriate for selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs), both being metabolized at an accelerated rate via different CYPs during pregnancy. Dose adjustments in pregnancy should be based on an individual assessment of symptoms, and determination of plasma levels with therapeutic drug monitoring (TDM) is recommended.

2.1.2 Challenges

It is obvious from the Subsection 2.1.1 that those physiological changes affecting pharmacokinetics are complex and variable. Manufacturers rarely provide dosing information in pregnancy because drugs are often not approved for use in pregnancy and are therefore used off-label. This accounts for the lack of robust pharmacokinetic data.

Pharmacotherapy in pregnancy is difficult and demanding: the therapy must be efficient for the mother and have as few side effects as possible; but must not harm the expected child. Caution is especially necessary in the first trimester, when the susceptibility to toxic effects on the developing organ systems and thus the risk of teratogenic damage is particularly high. The effects of drugs administered to the mother on the fetus are determined by the passage of the substances through the placental barrier.

2.2 Human placenta

The human placenta is an organ with an extremely complex anatomy and a unique physiology. In the following sections, its formation, functions, and transport mechanisms will be discussed to understand the diversity of existing *in vivo*, *ex vivo*, and *in vitro* models.

2.2.1 Human placental development and anatomy

Four days after fertilization, the morula (embryonic stage with 16 cells) develops into the blastocyst (32-64 cells). The placental development begins 6-7 days post-conception with the implantation of the blastocyst into the maternal uterine epithelium. The blastocyst consists of an inner cell mass which forms the fetus and fetal membranes and an outer single layer of mononucleated trophoblast cells which form the placenta. When the blastocyst has firmly attached (day 8 post-conception), some mononucleated trophoblast cells start to differentiate into the outer multinucleated syncytiotrophoblast, forming a complete mantel surrounding the conceptus. The remaining inner mononucleated trophoblasts are now referred to as cytotrophoblast, are not in contact with the maternal tissues, but actively proliferate and steadily fuse with the syncytiotrophoblast. The latter are responsible for the production of beta-human chorionic gonadotropin (β-hCG) by the second week post-conception, which is an indicator used in pregnancy testing. On day 9, lacunae or spaces form within the syncytiotrophoblast, allowing maternal blood from the uterine spiral arteries to enter the lacunar network, and an early uteroplacental circulation develops by the end of the 2nd week. These lacunae develop into intervillous space where the maternal blood leaves through endometrial veins. The remaining syncytiotrophoblastic mass between the lacunae (known as trabeculae) are of great importance for the further development of the villous trees of the placenta, as they begin to develop the first side branches at about day 13 post-conception (primary chorionic villous). Subsequently, these syncytiotrophoblasts mature into secondary villi by growing of extraembryonic mesoderm into these villi, forming a core of loose connective tissue. The development of first placental vessels by the end of the third week transforms the respective villi into tertiary villi. The latter either grow outwards into the intervillous space (branching villi), which then provides a large surface area for exchange between mother and fetus, or reach and contact the basal plate (anchoring villi) [4, 5]. The fetal-maternal barrier is relatively thick in the first pregnancy trimester (week 1-12 of gestation), since it then consists of the endothelial cells of the fetal capillaries and two continuous layers of trophoblast cells – an inner layer of cytotrophoblasts and an outer layer of multinucleated syncytiotrophoblasts. This layer gets thinner during the progression of pregnancy and, at the same time, the number of fetal capillaries rises. In the third trimester (week 28 of gestation until birth) this fetal-maternal barrier decreases to only a few cytotrophoblasts and an outer layer of multinucleated syncytiotrophoblast [6].

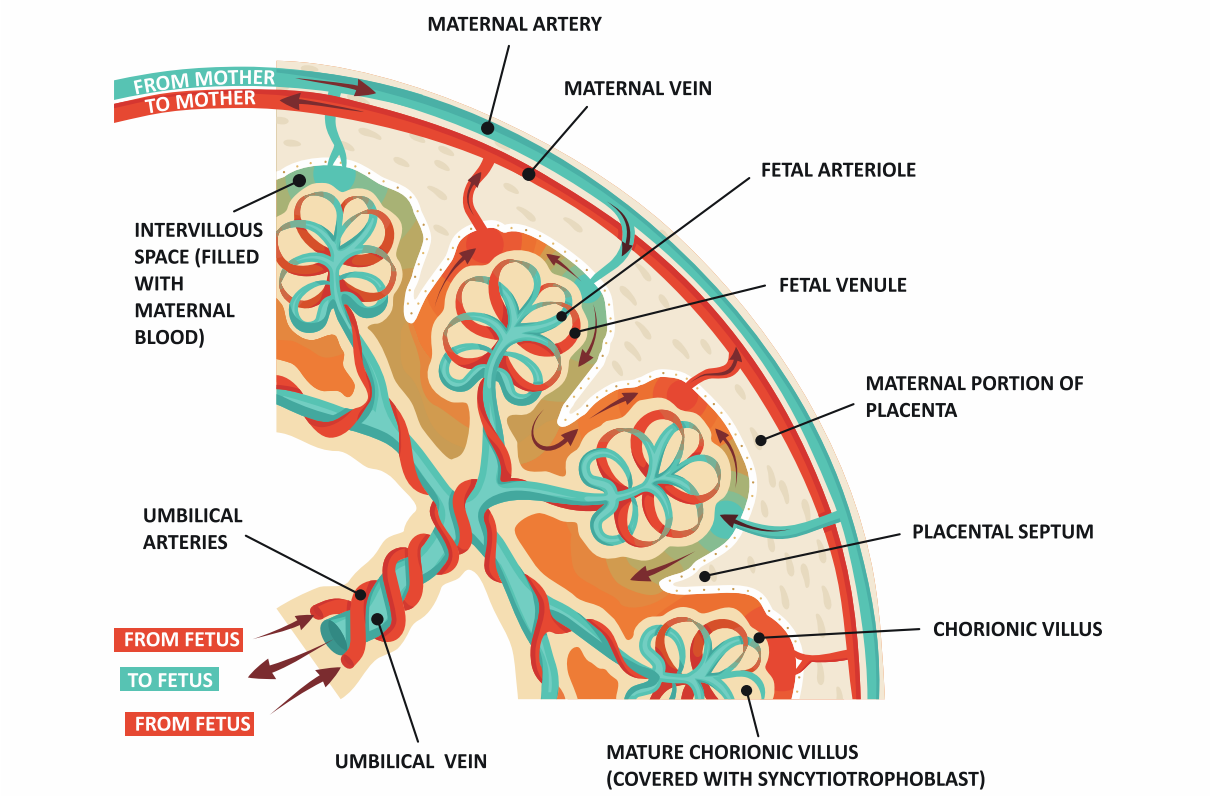


Figure 1. Anatomy of the placenta.
Photo source: logika600/Shutterstock [7]

The full-term placenta **(Figure 1)** is a discoid circular organ which is torn from the uterine wall at birth. At term, the average placenta has a diameter of 22 cm, thickness of 2.5 cm, and weight of 470 g. Nevertheless, there are considerable variations from placenta to placenta. The fetal surface (chorionic plate) is covered by the amnion – a single layered epithelium and the amnionic mesenchyme – and is covered by chorionic vessels (arteries and veins) that converge toward the umbilical cord, which is usually attached in the middle of the placenta. The maternal surface (basal plate) has 10-40 bulging regions called cotyledons, and the grooves correspond to the placental septa inside the placenta. Each maternal cotyledon is occupied by one to four villous trees and provides an average exchange area of up to 12.6 m^2 at term [8]. The chorionic and basal plates merge at the placental margin and form the fetal membranes which are composed of the amnion, the chorion, ant the decidua capsularis [4].

The structure of the human placenta is very different from that of other mammals. A distinction is made between the hemochorial (e.g., in humans, rodents, primates), the endotheliochorial (e.g., in dogs, cats) and the epitheliochorial (e.g., in pigs, horses, sheep) placenta. A placenta is termed hemochorial when the maternal blood in the intervillous space is in direct contact with the fetal trophoblast. In the endotheliochorial placenta, the two are separated by an additional layer of maternal endothelial cells or, in the case of the epitheliochorial placenta, by a layer of uterine epithelial cells. More specifically, the human placenta is a haemomonochorial one

because it has a single trophoblast layer at the time of birth. In contrast, the mouse placenta has up to three trophoblast layers, making it a haemotrichorial placenta. These and other anatomical and functional differences can lead to disparities between species, for example, in the transport of xenobiotics [8, 9].

2.2.2 Physiological function

The placenta is a highly specialized organ that plays a crucial and essential role during the nine months of pregnancy, performing various functions for the proper growth and development of the fetus. It supplies the developing fetus with blood, nutrients, and oxygen via the umbilical cord, while also regulating the removal of waste products and carbon dioxide. The placenta also protects the fetus from certain xenobiotics, infections ascending the reproductive tract, and maternal diseases. In addition, it is capable of metabolizing substances and releasing hormones into the maternal or fetal circulation that influence the course of pregnancy, fetal metabolism and growth, and labor itself [10]. The consequences of any placental dysfunctions are detrimental to the fetus.

Transport function

The main role of the placenta is to nourish and protect the sensitive developing fetus. Since the lungs of the fetus are still maturing *in utero* and thus do not participate in gas exchange, the placenta is entirely responsible for the transfer of oxygen and carbon dioxide to and from the fetus via the maternal blood supply. In contrast, the placental barrier also serves to protect the unborn, as the latter is particularly vulnerable to potentially toxic exogenous substances and to disturbances of homeostasis. This is regulated by various active and passive transport mechanisms (see chapter 2.2.3) located in the syncytiotrophoblast layer and the endothelial layer of the fetal vessels. Among the most important are the transport of glucose, since the fetus has a small capacity for gluconeogenesis, amino acids for fetal protein synthesis, of electrolytes, vitamins, water, and fatty acids. The latter are needed for the production of compounds that are important in cell signaling (e.g. prostaglandins), as well as for the production of biological membranes and myelin [11].

Endocrine function

The placenta secretes a palette of endocrine, paracrine and/or autocrine factors/hormones such as hCG, human placental lactogen (hPL), leptin, placental growth hormone (PGH), corticotropin-releasing hormone (CRH), gonadotropin-releasing hormone (GnRH), steroid hormones (such as estrogens and progesterone), and growth factors including epidermal growth factor (EGF), insulin-like growth factors (IGF), and platelet-derived growth factor (PDGF) [10]. The hormone hCG is produced by the syncytiotrophoblasts early in pregnancy. It stimulates the *corpus luteum* to secrete

progesterone, which is of great importance for the continuation of the pregnancy. Furthermore, the differentiation of cytotrophoblasts into syncytiotrophoblasts is stimulated by hCG (as well as by estradiol and glucocorticoids), to promote a normal placental development [10, 12]. The hormone hPL, that is also known as human chorionic somatomammotropin, is produced by the syncytiotrophoblasts. The hormone modifies the maternal lipid and carbohydrate metabolism throughout pregnancy to facilitate the energy supply of the fetus. In this context, it also reduces the insulin sensitivity of the expectant mother in order to increase the maternal blood glucose levels [11]. Leptin is another important signaling molecule of the reproductive system, secreted mainly by adipose tissue, but also by the trophoblastic cells of the placenta. Leptin seems to play a crucial role especially in the first stages of pregnancy, as it modulates many critical processes (proliferation, protein synthesis, invasion, apoptosis) of placental cells and is therefore essential for normal placentation and fetal development. However, it also helps to promote breast development for later milk production. Deregulation of leptin levels has been associated with various serious outcomes, such as recurrent miscarriage, intrauterine growth restriction (IUGR), gestational diabetes mellitus (GDM), and preeclampsia [13, 14]. Progesterone production begins in early stages of pregnancy and increases before labor to prevent uterine contractions and the onset of labor. Estrogens, on the other hand, stimulate the growth of the uterus and promote the development of the mammary glands [11].

Metabolic function

The placenta is not merely known for its transport and endocrine function, but also as a detoxifying and metabolizing unit of xenobiotics. Various CYPs are known to be present as phase I enzymes in the placenta, with type and amount of expression highly dependent on the maternal health status and gestational period. More CYP isoforms are expressed in the first trimester, when the fetus is most susceptible to the effects of teratogens, than at term. In contrast, only a few phase II enzymes are present [8, 15].

Immunologic function

Finally, the placenta also features an immune function, which plays a crucial role in the establishment, maintenance, and completion of a healthy pregnancy [16]. Although size is a limiting factor for proteins to cross the placental barrier, maternal Immunoglobulin G (IgG) antibodies can be transferred from the mother to the fetus by pinocytosis (see Subsection 2.2.3). This transfer begins in early gestation and increases exponentially towards the end of pregnancy to provide passive immunity to the baby in the first months of life [11].

2.2.3 Transport mechanisms

Until the 1960s, it was assumed that the placenta is a perfect barrier against harmful environmental agents, which was disproved with the thalidomide tragedy [17]. Today, it is known that the syncytiotrophoblast is the rate-limiting barrier separating the maternal and fetal circulation of the human placenta. Various transporters and enzymes are located at the apical and basolateral membranes of the syncytiotrophoblast (***Figure 2***), which regulate the exchange of nutrients between mother and fetus – including oxygen, carbon dioxide, and water – and eliminate metabolic waste products and hazardous chemicals from the fetus [11, 18, 19].

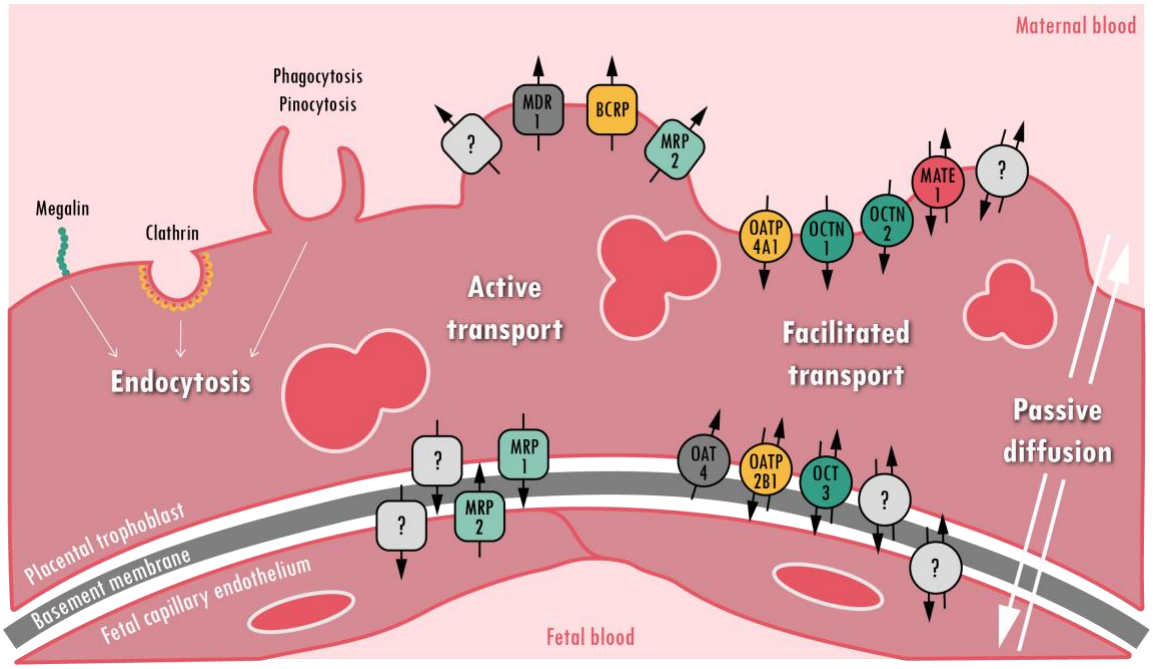


Figure 2. Transport processes within the human placental barrier (adapted from [18]).
BCRP: breast cancer resistance protein; MATE1: multidrug and toxin extruding protein 1; MDR1: multidrug resistance protein 1; MRP1: multidrug resistance-associated protein 1; 2: multidrug resistance-associated protein 2; OAT4: organic anion transporter 4; OATP2B1: organic anion transporting polypeptide 2B1; OATP4A1: organic anion transporting polypeptide 4A1; OCT3: organic cation transporter 3; OCTN1: novel organic cation transporter 1; OCTN2: novel organic cation transporter 2

Passive diffusion

Most drugs such as paracetamol and midazolam pass through the placenta via passive or simple diffusion, which occurs either transcellularly or paracellularly via aqueous channels that penetrate through the syncytiotrophoblast layer. This process occurs without an energy input as molecules are moved along a concentration gradient from areas of high to low concentration. This transport mechanism is not saturable and does not participate in competitive inhibition. However, the drug transfer across the placenta via passive diffusion depends on several physical and pharmacological factors such as placental surface area/thickness, pH of maternal and fetal blood, placental metabolism, molecular weight of the drug, lipid solubility (pKa), degree of ionization, protein binding, and concentration gradient across the placenta [8, 11, 20, 21].

Facilitated diffusion

Similar to passive diffusion, facilitated diffusion occurs along a concentration gradient whereby no energy input is required. The main difference is that facilitated diffusion is carrier-mediated and requires a transport protein (carrier) for the transfer across the placental membrane. This process is inhibited when the carrier molecules are saturated by the substance itself, by competitive substrates, or by endogenous substrates competing for their use. Substances that are structurally related to endogenous compounds are often transported by facilitated diffusion (such as glucocorticoids or cephalosporins) [8, 11, 20, 21].

Active transport

Active transport is also carrier-mediated and saturable, with competition between related molecules. The difference to other transport processes is that energy, usually in the form of ATP, is used to transport substances against a concentration or electrochemical gradient. The required energy can also be obtained from energy stored in the transmembrane, which is classified as secondary active co-transport and occurs through an ionic electrochemical gradient provided by H^+, Na^+, or Cl^- [19]. The transporters are located either on the fetal basolateral side (mostly uptake carriers), or on the maternal membrane of the syncytiotrophoblast (mostly efflux carriers) [21], allowing essential nutrients such as amino acids, vitamins, iron, and calcium to be transported to the fetus, and xenobiotics and other undesirable substances to be removed from the fetal circulation. A variety of active transporters have been identified in the placenta, including the expression of members of the P-glycoprotein (P-gp) and multidrug resistance proteins family in the human term placenta [8, 11, 20-22].

Endocytosis

Endocytosis consists of different mechanisms: pinocytosis (energy-required liquid phase endocytosis), phagocytosis (engulfment and destruction of extracellular material) and receptor-mediated endocytosis. The latter consists of internalization of selective extracellular ligands (e.g., IgG- or IgG-based antibodies) into cytoplasmic vesicles, with the rate of transfer depending on several factors, such as membrane fluidity, vesicle mobility in the cytosol, and the rate of receptor turnover [21]. All mechanisms are driven by intracellular membrane vesicles to release the transported substance (e.g., nanoparticles or liposomes) on the opposite side. However, endocytosis is a minor route of mass transfer for drugs, as this process is too slow to exhibit relevant drug concentration on the fetal side of the placenta [8].

2.2.4 Models to study placental transfer

Complex questions arise when administering drugs to pregnant women in terms of placental drug transfer and metabolism. Pregnant women are usually excluded from clinical trials due to ethical aspects. It is therefore understandable why pharmaceutical companies generally label drugs as contraindicated or not approved for pregnant women (or during breastfeeding) [8]. Therefore, a variety of models have been developed for studying the placental transfer and metabolism of substances/xenobiotics. These models partially resolve the ethical and methodological problems, but they are not without limitations. *In vitro* models cannot fully account for the multitude of physiological changes in the mother, placenta, and fetus which occur throughout the pregnancy, while *in vivo* studies prevent a direct extrapolation of results because of anatomical and functional differences between mammalian placentas [8].

In vivo models

Animal models

Many placental transfer studies have been performed in rodent models, such as in mice and rats [23], *in situ* placental perfusion techniques have been established in small animals (guinea pigs, rabbits), and chronically cannulated sheep have been used extensively [24]. However, for all animal models, extrapolation of the results to humans is limited due to large interspecies differences. The placenta of non-human primates, such as rhesus monkeys, is the most similar to the human placenta, as the maternofetal tissue layers are also classified as haemomonochorial according to the Grosser classification, and the macrostructure and maternofetal blood flow are villous and multivillous, respectively [20, 24]. Additionally, animal experiments pose ethical and financial hurdles in addition to the aforementioned limitations [25].

Fetal/maternal blood concentration ratio

Determination of the ratio between fetal and maternal blood concentrations is the simplest and most defensible index for placental transfer *in vivo*. For this purpose, blood samples are taken simultaneously from a peripheral vein (maternal blood) and from the umbilical cord (fetal blood). Many studies have estimated this ratio after administration of a single dose, which may not be a reliable estimate because it may lead to ratios with a high variability. More meaningful information about the drug distribution between those two circulations can be obtained by maternal drug infusions (starting with a loading dose) to maintain a constant maternal drug concentration [8]. Disadvantages of this method are the difficulties in assessing the influence of placental metabolism on drug transfer and the lack of information on tissue distribution and accumulation [15]. The

method can be further expanded by performing placenta biopsies to investigate the maternal-placental or fetal-placental concentration ratios [26].

Coelocentesis

This technique was introduced in 1991 as an alternative to fetal blood sampling. It involves sampling from human exocoelomic and amniotic fluid via transvaginal puncture early in pregnancy (weeks 5-13). This method is suitable for the study of placental transfer, but not for the kinetics of compounds [26-28].

Ex vivo model

Human placental perfusion

The current gold-standard among the placental transfer models is the *ex vivo* human placental perfusion **(Figure *3*)** using placentae that are obtained immediately after delivery (both vaginal births and cesarean sections) [29, 30].

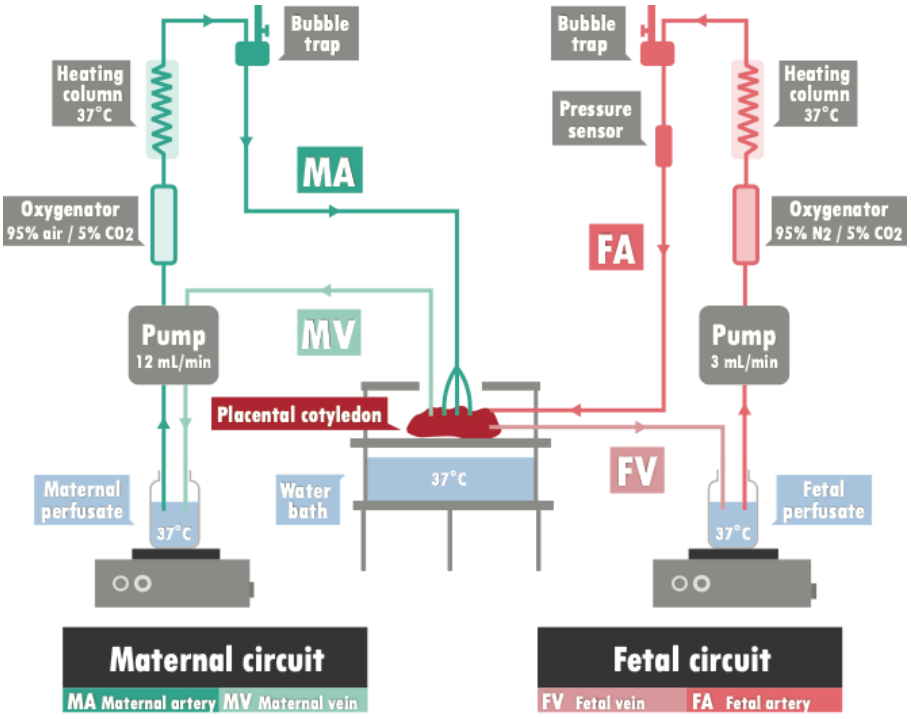


Figure 3. *Ex vivo* placental perfusion model to investigate transplacental transfer.

The model was first described by Panigel et al. [31] in 1967 and was further developed by Schneider et al. [32], and since its introduction the number of perfusions has steadily increased [24, 26]. Advantages of this model are that the structure of the cotyledon is fully preserved [33], the cells in

perfused placentae remain technically viable for up to 48 h [26], and data obtained are highly predictive of the *in vivo* transfer [34]. Furthermore, the procedure is non-invasive for both, the mother and child, which creates a minimum of ethical issues. In addition to information about the transplacental transfer, the model also offers investigation of placental metabolism and fetal metabolites, preferential direction of transport and the presence and involvement of transporters, and acute toxicity [26]. The main drawback of this model is that the placenta only reflects the situation at the end of a pregnancy, and not that of the first trimester in which the susceptibility of the fetus to toxic hazards is high. However, it is possible that the placenta might be more sensitive to environmental substances at term, as the cell layer thickness decreases toward the end of pregnancy. In addition, the involvement of placental transport mechanisms and metabolism within the tissue may be underestimated because they have run down at the time of delivery compared to first trimester placentae [33]. Due to interindividual variation, a large number of placentae may need to be perfused to obtain reproducible results, which is very time and labor intensive [15, 24]. The method itself is described in Chapter 4.

Non-human placental perfusion

Ex vivo perfusion models of the rodent placenta (rats [35] and mice [36]) have also been used for an early screening of substance transfer across the placental barrier, again raising the issue of comparability to the situation in pregnant women.

In vitro models

In vitro models utilizing well-established trophoblast-derived immortal human placental cell lines (e.g., BeWo, JAR, JEG-3 cells) which originate from choriocarcinoma cells or human placental primary cells (villous trophoblasts) and explant tissue have been employed. These latter models enable the study of various factors affecting the transplacental transport, such as uptake, efflux, and metabolism. Furthermore, they can replace or reduce the number of animal testing and elude the ethical and methodological hurdles of *in vivo* studies [25]. However, all cell-based placental models lack the cellular organization, compartmentalization and 3-dimensional structure of intact, physiologically active placentae [37].

Placental cell models

The classical approach to model the placental barrier *in vitro* is the Transwell system **(*Figure 4*)**, which has been widely used to study the transport of a variety of endogenous and exogenous compounds [38, 39]. Furthermore, the model is used to study trophoblast differentiation [40], placental metabolism, and nutrient and drug distribution across the placental barrier [15].

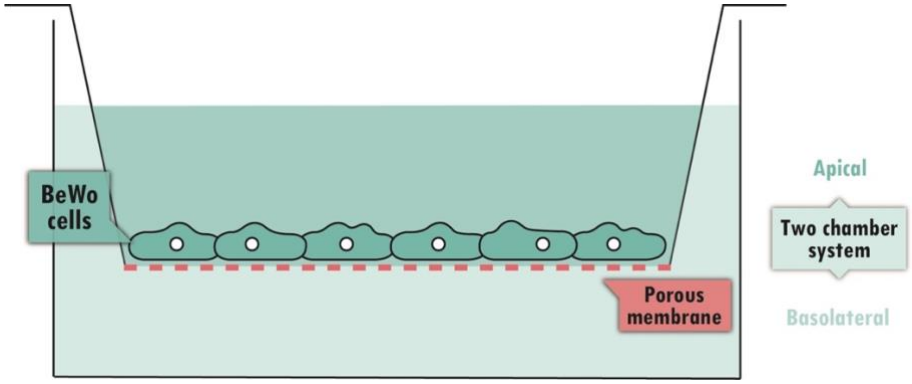


Figure 4. *In vitro* Transwell model with BeWo b30 cells to investigate transplacental transfer.

Cells (e.g. BeWo b30) are thereby cultured on semi-permeable membrane inserts until they form confluent monolayers. The BeWo b30 cell line is a clone of the BeWo cell line which was originally derived from a human choriocarcinoma and has been most used. The original BeWo cell line, however, is controversially discussed if it has the same monolayer-forming abilities in culture as the BeWo b30 clone [41, 42] and may therefore be less suitable as a representative model of transepithelial barrier. Other human trophoblast-derived cell lines such as JAR, JEG-3, and ACH-3P have differences in cellular function and should be chosen carefully depending on the original research question [43]. The value of cell lines as a model highly depends on their similarity to normal trophoblastic cells. However, the isolation of primary cytotrophoblasts from the human placenta brings a high risk for contamination, and the cells are not viable for many passages [44]. Moreover, these do not form a confluent monolayer [45]. In general, the cell lines are comparatively easy to handle, and the Transwell model is suitable for long-term exposure studies. In addition, a good correlation between results of *ex vivo* perfusion of human placenta and *in vitro* BeWo b30 cells in the Transwell model was demonstrated with several model substrates [38, 46]. The major drawbacks of the experimental model are the absence of blood flow and the simplification of the cellular complexity of a placenta. For the latter reason, more complex Transwell models have been developed in which epithelial cell lines such as pure vascular endothelial cell line from the human placenta (HPEC) and human umbilical vein endothelial cells (HUVECs) are used for coculture with the trophoblast cell lines to better reflect the physiological situation *in vivo* [25, 47, 48]. As an alternative, 3D cell models can be used to study the human organ physiology, allowing the cells to self-organize into spheroid- or organoid-like structures. Finally, there are placenta-on-a-chip (PoC) models are relying on microfluidic channels to generate flow.

Placental tissue explants

Placental tissue can be cultured *in vivo* as tissue explants to investigate the transport of substances from the maternal circulation into the syncytiotrophoblast, as well as the metabolism of xenobiotics, enzyme and endocrine function, cellular proliferation, and differentiation. The tissue can be obtained from different stages of gestation (early vs. terminated pregnancy) and can be in culture for up to 11 days. Other advantages include the intact microarchitecture and maintenance of cell-cell interactions and paracrine communications in explants. Disadvantages of this model are that (i) the explant culture is very sensitive to environmental influences and therefore needs to be closely monitored, (ii) the blood flow cannot be simulated, and (iii) it is difficult to perform good kinetic transfer studies [44, 49, 50].

Placental membrane vesicles

This model allows the study of the expression and functionality of specific transporters of the fetal as well as maternal plasma membranes since membrane vesicles can be isolated in a relatively purely form from the brush border or from the fetal-facing basal membrane of trophoblasts. However, these plasma membrane vesicles lack intracellular components, which limits the study of regulatory processes using signaling cascades, and an estimation of the transporter activity contribution *in vivo* is not possible [15, 51].

In silico models

Several types of *in silico* models are available to predict different aspects of placental physiology and pathology. The physiologically based pharmacokinetic (PBPK) and quantitative structure-activity relationship (QSAR) model, for example, focus on the barrier transport of xenobiotics. These models have no ethical or financial constraints [25].

2.3 Mild mental disorders and/or symptoms in pregnancy

Two main classification systems are used to diagnose mental disorders: the 10th revision of the International Classification of Diseases (ICD-10) [52], which is published by the World Health Organization (WHO), and the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) [53]. These two classification systems cover a wide range of relevant mental disorders. However, this work focuses on NMDs, which include mood disorders, anxiety disorders, and sleep disorders. They are often less severe than psychotic disorders, and symptoms depend on the disorder, but may include agitation, depressed mood, irritability, lack of energy, restlessness, social withdrawal, and insomnia among others [54].

2.3.1 Prevalence

Pregnancy is a vulnerable period for NMDs. This was illustrated by a meta-analysis that included thirteen publications from seventeen different (lower-middle-income) countries which reported a prevalence of 15.6% in pregnancy [55]. A population-based study of pregnant women during the second trimester at two obstetric clinics in Sweden found, that psychiatric disorders were present in 14.1% of the women, among of which only 5.5% were being treated [56]. This has also been observed in the USA using a screening questionnaire, where 20.0% of pregnant women showed a depressive symptomatology, with only 13.8% reporting a formal treatment for depression [57]. Comparable data exist in Switzerland, where the annual rate of perinatal women using mental health services accounts for 16.7% [58], clearly underlining the vulnerability of pregnant women to mental disorders.

2.3.2 Treatment

Antidepressants

The mainstay of pharmacological treatment for NMDs in the perinatal period is antidepressants **(Table *1*)**. The SSRIs are the most used and researched (1st option), followed by serotonin-norepinephrine reuptake inhibitors (SNRIs; 2nd option) and TCAs [59, 60].

Table 1. General overview of different types of antidepressants with some representatives registered on the Swiss pharmaceutical market [61].

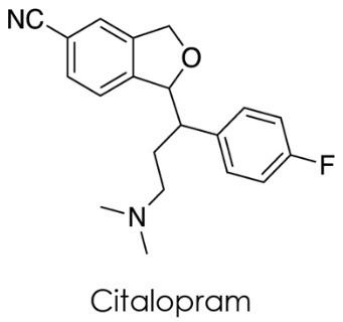
SSRI	SNRI	TCA	MAOI	Others
Citalopram Escitalopram Fluoxetine Fluvoxamine Paroxetine Sertraline	Duloxetine Venlafaxine	Amitryptyline Clomipramine Dibenzepin Doxepin Imipramine Trimipramine Opipramol	Moclobemide	Agomelatine Bupropion Lithium Mianserin Mirtazapine Reboxetine Trazodone

SSRI = selective serotonin reuptake inhibitor; SNRI = serotonin-norepinephrine reuptake inhibitor; TCA = tricyclic antidepressant; MAOI = monoamine oxidase inhibitor

Selective serotonin reuptake inhibitors

In general, SSRIs are the first choice for mild to moderate depression and may also be used for anxiety disorders, panic disorders, or obsessive-compulsive disorders. Possible side effects may include gastrointestinal symptoms (e.g. nausea), insomnia and agitation, or sexual dysfunction [61].

The results on their influence on pregnancy outcome are contradictory. To date, most studies associated the exposure to SSRIs during pregnancy with preterm birth, lower birth weight, and withdrawal symptoms in infants among others (see [62] for additional references). A neonatal adaptation syndrome (NAS) with irritability, abnormal crying, agitation, lethargy, tremor, feeding difficulty, and dyspnea is well-documented and occurs in up to 30% of exposed infants [59, 63]. In addition, the risk of congenital malformations is increased [64-66], and specifically cardiac malformations have been reported [66]. On the other hand, a large cohort study failed to demonstrate an association between SSRIs and the increase of cardiac malformations [27]. Comparing different guidelines on treatment of perinatal depression with antidepressants, citalopram **(Figure *5*)** and fluoxetine are mentioned as preferred medication, although their use during pregnancy remains controversial [67]. Late pregnancy exposure to SSRIs, as citalopram, is associated with poor neonatal adaptation syndrome, including symptoms such as constant crying, respiratory difficulty, eating and sleeping difficulties, increased muscle tone, seizures and irritability in general [68].



Citalopram

Figure 5. Chemical structure of citalopram – a selective serotonin reuptake inhibitor (SSRI)

For paroxetine, an association between the use during pregnancy and congenital heart defects has been demonstrated [64], and it should be avoided in the first trimester, despite the very low risk (see [59] for additional references). For persistent pulmonary hypertension, a rare but fatal disorder in up to 15%, the risk is slightly increased for all SSRIs (incidence 0.3% versus 0.12%) [69, 70].

Serotonin and norepinephrine reuptake inhibitors

In general, if SSRIs are not sufficiently effective, SNRIs may be an alternative treatment option, although gastrointestinal symptoms (e.g., nausea), insomnia, and restlessness or sexual dysfunction may also be among the possible side effects. However, compared with SSRIs, side effects and side-effect-related discontinuations are more common among SNRIs [61].

There is insufficient information available for SNRIs in pregnancy with regard to long-term effects, and they must therefore be used with caution and after careful consideration (benefits vs. risks) [59, 71]. A comprehensive cohort study (sibling controlled design) failed to show an association between *in utero* venlafaxine exposure and the increase for cardiac malformations [72].

Tricyclic antidepressants

In general, TCAs block the reuptake and thus inactivation of norepinephrine and serotonin presynaptically. They have more side effects than SSRIs, particularly sedative and anticholinergic ones (such as dry mouth and constipation), orthostatic hypotension, and weight gain. In overdose, these agents can cause moderate to high lethality [61].TCA exposure in pregnancy slightly increases the risk for complications (preterm birth and low birth weight), general malformations, and cardiovascular defects during pregnancy compared to SSRIs [64].

Anxiolytics

Benzodiazepines

The first choice for women to treat anxiety disorders are antidepressants (SSRIs and SNRIs), although benzodiazepines (e.g., alprazolam, diazepam, or lorazepam) may also be useful if necessary. In addition, benzodiazepines and other hypnotics (non-benzodiazepines) may also be useful for intermittent insomnia [59]. The anxiolytic effect of benzodiazepines is immediate and, in contrast to antidepressants, does not lead to restlessness and nervousness at the beginning of treatment. Because of the potential for dependence, the fast tolerance development, and the occurrence of withdrawal symptoms on discontinuation, treatment with benzodiazepines requires an adequate risk-benefit assessment. In addition, long-term use is not recommended (maximum 4

weeks) [73]. Diazepam **(Figure *6*)** is a particularly well-known benzodiazepine and is often prescribed during pregnancy. While diazepam use during gestation has no adverse effects on the development of the exposed child, its use in the weeks before delivery may provoke neonatal withdrawal syndrome, floppy infant syndrome, or various acute toxic effects in the newborn [74].

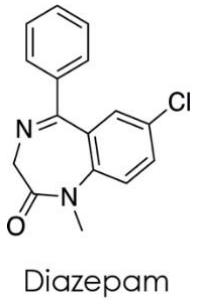


Figure 6. Chemical structure of diazepam – a benzodiazepine

Hypnotics

Hypnotics (non-benzodiazepines) such as zolpidem or zopiclone may be useful for the treatment of intermittent insomnia. However, the fetal risks of the active pharmaceutical ingredients have not been equally well studied to date and should therefore be well weighed against the benefits and/or alternatives [59].

As shown, treatment of mental diseases with conventional medications during pregnancy has several disadvantages. In a questionnaire distributed to pregnant women in obstetric clinics in the Canton of Zurich, only a very small number of participants reported the use of sedatives/anxiolytics or antidepressants during pregnancy in comparison to before getting pregnant [75]. However, untreated NMDs themselves should be categorically avoided. For instance, untreated depression during pregnancy is linked to poor birth outcomes (preterm birth, low birth weight, fetal intrauterine growth retardation), and it can have a variety of negative effects on women (disrupted sleep, increased risk of suicide, increased exposure to illicit drugs). Furthermore, untreated major depression during pregnancy is the most robust predictor of postpartum depression [76-79]. Based on these arguments, we investigated the safety of various herbal medicines which may be promising options to treat mild NMDs in pregnancy.

2.4 Herbal medicine

2.4.1 Historical background

Herbal medicines represent the oldest source of pharmacotherapy, and archaeological evidence revealed that the use of medicinal plants dates to the Paleolithic age approximately 60'000 years ago. The earliest records about the medicinal use of over 250 various plants were from Mesopotamia (2600 BCE) written on clay tablets, followed by the best known Egyptian pharmaceutical record *Ebers Papyrus* (1550 BCE) which contains about 700 herbal medicines including their dosage forms. [80, 81]. Dioscorides (1st century AD), is known as the "father of pharmacognosy" and the important author of the work *De Materia Medica* (77 AD), which includes a description of 944 drugs (657 of which are of plant origin). The descriptions include information on the appearance, place of discovery, occurrence/cultivation, collection mode, medicinal preparation, and therapeutic effect. This work of ancient history, together with the book *Historia naturalis* by Pliny the Elder (23-79 AD), which includes over 1000 medicinal plants, was the basis of knowledge on medicinal plants until the late Middle Ages and the Renaissance.

The term *phytotherapy* was introduced into medical science only later by the French physician Henri Leclerc (1870-1955). Modern phytotherapy is not part of alternative medicine and is fundamentally different from homeopathy and anthroposophic medicine. In contrast to conventional pharmacotherapy, phytotherapy only uses active ingredients that consist of plant parts (e.g. flowers, roots), constituents (e.g. essential oils) or their preparations (e.g. dry extracts, tinctures, pressed juices) [82].

Nowadays, according to the WHO, about 75% of the world's population rely on herbal medicines for primary health care [80]. In addition, the phytochemicals present in herbs are being actively investigated for the development of new (synthetic) drugs and are therefore an important and indispensable source in drug discovery [83]. For instance, in 2001, a total of 122 compounds/structures from 94 different plant species were identified which are nowadays used as drugs worldwide [84].

2.4.2 Regulatory aspects

National regulations

In Switzerland, a marketing authorization is required for herbal medicines with specified indications, and the quality, safety and efficacy must be demonstrated. The regulatory requirements for safety assessment are the same as for conventional pharmaceuticals. Sufficient is either a simplified marketing authorization procedure for traditional use of the herbal medicines (without proven adverse effects) or, where appropriate, clinical trials may be replaced by scientific literature or proof of pharmaceutical or therapeutic equivalence with an already approved product to demonstrate efficacy and safety [85, 86].

Regulation in the European Union

Committee for Herbal Medicinal Products monographs

With the European Union (EU) Directive 2001/83/EC Article 10a, the possibility was created to obtain a marketing authorization for herbal medicinal products only based on safety and efficacy data from the literature, which is referred to as "well-established use". However, since there are also many products that have a long tradition of use (≥ 30 years), but whose efficacy is difficult to substantiate by clinical studies or literature evidence according to the current requirements, Directive 2002/24/EC established the possibility of facilitated registration as a traditional herbal medicinal product ("traditional use") in the European directive system. Overall, however, the efficacy justification including dosage and dosage form must be plausible. The Committee for Herbal Medicinal Products (HMPC) was established as part of the central regulatory authority EMA (European Medicines Agency), in which every EU member states are represented. Since 2008, the Committee has been reviewing and evaluating available scientific material and subsequently producing monographs that are intended to be binding for the regulatory authorities in all countries of the EU [82].

European Scientific Cooperative on Phytotherapy monographs

The European Scientific Cooperative on Phytotherapy (ESCOP) was founded in 1989 as an umbrella organization representing national herbal medicine and phytotherapy societies throughout Europe. ESCOP members include various EU Member States, as well as Switzerland and Turkey. It is primarily engaged with the establishment of herbal medicine monographs, which are based on published data and have a high scientific value, as they contain detailed information on: phytochemistry, use, dosage, side effects, contraindications, interactions, and results from

pharmacological, toxicological, and clinical studies. Another objective of ESCOP is to harmonize the regulatory status (marketing authorization) of herbal medicinal products within Europa [82].

World Health Organization monographs

The monographs of the WHO serve to harmonize the quality and the traditional and modern clinical use of medicinal plants internationally. They are intended to support the development of national formulations and to help developing countries regulate their pharmaceutical markets. The selection of drugs is therefore primarily based on those used worldwide with common indications [82].

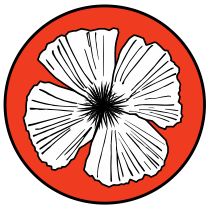
2.4.3 Use in pregnancy

Midwives are the primary practitioners of complementary and alternative medicine (CAM) during pregnancy, particularly in the areas of homeopathy, acupuncture and phytotherapy, which are of significant importance as 43.2% (205/475) of women undergoing pregnancy choose CAM treatment [87]. A German study reported that about one third of pregnant women take herbal medicines with regard to the treatment of psychological problems [88]. Use of herbal medicines in pregnancy was also reported in a multinational study (28.9%, 2'735/9'459), with significant geographic variation (e.g., 40.6% in Switzerland) and mostly self-medicated. The five most commonly used herbal medicines were ginger, cranberry, valerian, raspberry, and chamomile, although is in some cases little is known about their efficacy and safety [89]. Despite this lack, a variety of CAM therapies, including herbal medicine, are recommended to pregnant women by healthcare professionals involved in the care of pregnant women (e.g. midwifes, obstetricians, anesthetists) – much more often by those who use CAM themselves [90]. In contrast, a cross-sectional survey in the United States revealed that 50% of CAM users (including herbal medicine) reported not discussing its use with their obstetricians. Therefore, health professionals should actively inquire about the herbal medicines used and should be informed on safety issues [91, 92]. A number of reviews [93-97] and studies [98] have already addressed whether herbal medicines can be considered safe in pregnancy. They also point out the potential for interaction and toxicity of herbal medicines and emphasize that women and health professionals need to move away from the idea that herbal medicines are not harmful [99]. Alternative approaches to clinical trials on drug safety during pregnancy rely on information from toxicology centers [100] or pharmacovigilance services [101], both of which are valuable sources of information.

2.4.4 Potential plants used in the treatment of mild mental disorders

St. John's wort, California poppy, valerian, hops, and lavender are some well-known herbal medications for the treatment of NMDs **(Table *2*)**. Besides their prominence, they exert many pharmacological activities and some of their ingredients are of great interest.

Table 2. Detailed overview of plants used for the treatment of NMD including scientific name, drug parts used, typical ingredient(s) and clinical effects [82, 102], and recommended maximal daily dose of a representative medicinal product [61].

	Plant ***Scientific name***	**Drug**	**Selection of typical ingredient(s)**	**Main putative clinical effects or indications**	**Dose* (mg)**
	**St. John's wort** *Hypericum perforatum*	Herba	Anthracene derivatives, acylphloro-glucinols	Antidepressant, anxiolytic	900
	**California poppy** *Eschscholzia californica*	Herba	Isoquinoline alkaloids	Sedative, anxiolytic, spasmolytic	1200
	**Valerian** *Valeriana officinalis*	Radix	Iridoids, sesquiterpenes	Nervousness, insomnia	1750
	**Lavender** *Lavandula angustifolia*	Flos	Volatile essential oil, tannins	Anxiolytic, insomnia	80
	**Hops** *Humulus lupulus*	Strobulus	Acylphloro-glucinols, alpha-bitter acids	Sedative, nervousness, anxiolytic	120

* Representative medicinal product for St. John's wort (Jarsin®), California poppy (Arkocaps® Escholtzia), valerian (Arkocaps® Baldrian), lavender (Lasea®) and hops (Redormin®)

Other plant candidates

In addition to the plants mentioned above, there are a variety of plants that could also be considered for the treatment of NMDs due to their effects **(Table *3*)**. However, some of them have primarily different indications, such as hypertension or premenstrual symptoms.

Table 3. Summary of plants that exhibit anxiolytic, sedative, and/or antidepressant activities, among others [82].

Plant ***Scientific name***	**Drug**	**Selection of clinical effects or indications**
Cannabis *Cannabis sativa*	Summitates et resina	Psychotropic, euphoric, sedative, muscle relaxant, spasmolytic, analgesic, antiemetic
Chamomile *Matricaria chamomilla*	Flos	Antiphlogistic, wound-healing, spasmolytic, anxiety disorder (demonstrated experimentally in animals and clinically in double-blind study), mild forms of insomnia
Indian snakeroot, devil pepper or serpentine wood *Rauwolfia serpentina*	Radix	Mild essential hypertension, sedative, tranquilizer against nervous and mental disorders
Kava or kava kava *Piper methysticum*	Rhizoma	Anxiolytic, sedative, anticonvulsive, spasmolytic
Lemon balm *Melissa officinalis*	Folium	Calming, carminative, spasmolytic, for functional gastrointestinal complaints, nervous sleep disorders
Passion flower *Passiflora incarnata*	Herba	Motility inhibiting, anxiolytic, fear, insomnia
Pomegranate *Punica granatum*	Fructus	Antioxidant, hepatoprotective antiphlogistic, antiarteriosclerotic, antidepressant (experimentally proven in mice)
Sage *Salvia officinalis*	Folium	Antibacterial, fungistatic, virustatic, astringent, antiphlogistic, according to WHO monograph also as sedative
Vitex or chaste tree *Vitex agnus-castus*	Fructus	Premenstrual syndrome, menopausal symptoms, Restless Legs Syndrome, according to WHO monograph also as sedative
Yohimbe *Corynanthe johimbe*	Cortex	Agitation, tremor, insomnia, anxiety

2.5 St. John's wort

St. John's wort (*Hypericum perforatum* L. **Figure 7**) belongs to the family of Hypericaceae and has many German synonyms such as Blutkraut, Johannisblut, Sonnwendkraut, and Hexenkraut. The plant is also called dotted or perforated St. John's wort, referring to the clearly visible oil glands (predominantly blood-red to black hypericin vessels) on the flowers and leaves, which is where the Latin species name *perforatum* comes from. *Hypericum*, however, comes from the Greek *hyperikon*, which is composed of the word roots "hyper" (over) and "eikon" (image, apparition), as the plant is supposedly warding off evil spirits. The herbaceous perennial plant, about 40-80 cm high, has bright yellow flowers with four to five petals, numerous long stamens, and a single pistil. The species is a native of Europe is nowadays mainly cultivated in Germany, Eastern Europe, and Chile, and is found in the wild only in small quantities [103-105].

Figure 7. St. John's wort (*Hypericum perforatum* L.)
Photo source: Scisetti Alfio/Shutterstock [106]

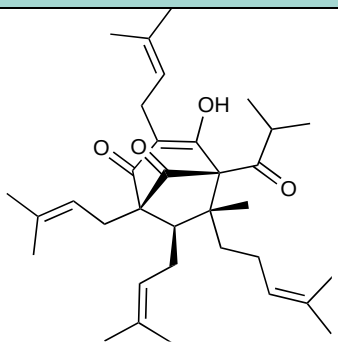
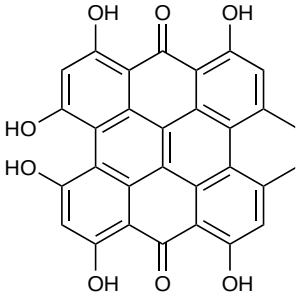
2.5.1 Historical use

St. John's wort has a long history as an antidepressant, antiseptic, anti-inflammatory, expectorant, and immune system booster. According to the first reports, the herb was used as early as the 1st century AD by the Greek herbalist Dioscorides, and later by the herbalist/alchemist Paracelsus (1493-1541) who recommended St. John's wort preparations to and alleviate pain and treat wounds [104]. Then, in 1525, Paracelsus suggested to use St. John's wort for the treatment of depression, melancholia and overexcitability [107]. The name of the plant refers to the birthday of St. John the Baptist on June 24. Christians probably gave the name to the plant, noticing that the herb is in full bloom at this time. Today, St. John's wort is one of the most popular remedies available in various forms (capsules, liquid extracts, oils, ointments, etc.) and is mainly used to treat mild to moderate depression. Interestingly, this was already recognized by Hildegard of Bingen (1098-1179), who said about the plant that it was a "suitable herb against black melancholy" [105, 108].

2.5.2 Phytochemistry

St. John's wort contains numerous groups of natural compounds with the naphthodianthrones, phloroglucinols, and flavonoids being the most common classes. The most extensively researched class of isolated compounds are the naphthodianthrones (drug contains 0.1-0.3%), the so-called hypericins, with the main components hypericin, pseudohypericin, protohypericin, and isohypericin. Of these, the highly photoreactive hypericin **(Table *4*)** is the best studied and is particularly found in the black dots in the flowers, being responsible for the red color of St. John's wort oils. Another class of compound are the lipophilic phloroglucin derivatives (drug contains 0.2-0.4%), with the main components hyperforin **(Table *4*)** and adhyperforin, as well as their degradation products. Hyperforin is unstable in the presence of oxygen and light [109]. Flavonoids (drug contains 2-4%) include flavones (luteolin), flavonols (kaempferol, quercetin), glycosides (hyperoside, isoquercitrin, and rutin), biflavones (biapigenin) and many more which are all biogenetically related. In addition, St. John's wort contains small amounts of essential oil, which mainly consists of sesquiterpenes (caryophyllene, spathulenol, etc.) [103, 104, 110].

Table 4. Physicochemical characteristics of hyperforin and hypericin [111].

Study compounds	Hyperforin	Hypericin
Chemical structure		
Empirical formula	$C_{35}H_{52}O_4$	$C_{30}H_{16}O_8$
Molecular weight (g/mol)	536.78	504.44
Hydrogen-Donors	1	6
Hydrogen-Acceptors	4	8
Rotatable bonds	11	0
Rings	2	8
Lipinski violations	2	3
Solubility in water (mg/mL, 25°C)	0.006 (highly insoluble)	0.0000002 (highly insoluble)
Partition coefficient log $K_{oc/w}$	10.22 (very lipophilic)	7.89 (very lipophilic)

2.5.3 Pharmacological activities

St. John's wort exerts a range of biological effects (e.g., anti-inflammatory, antibacterial, antiviral, anti-cancerous, antioxidant, neuroprotective), the most important of which is, of course, the antidepressant activity [110, 112]. The plant contains numerous bioactive compounds with potential neuroactive effects, including hyperforin and hypericin [113]. Research initially attributed the antidepressant effect to hypericin, which was found to inhibit monoamine oxidase (MAO) enzymes type A and B in rat brain mitochondria (significant inhibition only at very high hypericin concentrations) [114]. However, the effect is likely due to the combination of several plant constituents , including hyperforin, which is a potent reuptake inhibitor of neurotransmitters such as dopamine, norepinephrine, serotonin, γ-aminobutyric acid (GABA), and L-glutamate [115]. Hyperforin reveals a new mechanism of action, as it activates canonical transient receptor potential channel 6 (TRPC 6), causing the entry of calcium and sodium ions into cells, thereby reducing the cation gradient that controls monoamine neurotransmitter uptake. Thus, the mechanism of action of hyperforin differs from conventional antidepressants, which inhibit neurotransmitter transporter proteins directly [116, 117]. Additionally, the presence of different flavonoids, such as hyperoside, isoquercitrin, and rutin, may modulate the antidepressant effect of St. John's wort [118].

St. John's wort can interact with various medications, including antidepressants, oral contraceptives, immunosuppressants, and anticoagulants. The herb can reduce the effectiveness of these medications by increasing their metabolism and decreasing their blood levels [112, 119]. One of the primary compounds thought to be responsible for St. John's wort's interactions with certain medications is hyperforin [120]. Therefore, the risk of herb-drug interactions may be influenced by the choice of St. John's wort product, as the amount of hyperforin can vary widely [121].

2.5.4 Clinical evidence in the treatment of mild mental disorders

The antidepressant efficacy of extracts of St. John's wort has been confirmed in several clinical studies: A meta-analysis of twenty-seven studies revealed that the herbal antidepressant did not differ in efficacy from SSRIs, and treatment of mild-to-moderate depression with St. John's wort extract resulted in fewer adverse events and thus fewer treatment discontinuations [122]. This analysis included a study that enrolled 135 patients with a major depression disorder and found that a (hyperforin-rich) standardized extract of St John's wort was more effective than fluoxetine and placebo in treatment over a 12-week period [123]. St. John's wort extract was also therapeutically equivalent to imipramine – a TCA – in the treatment of mild to moderate depression, but was better tolerated by the 324 patients included, as shown in a randomized, multicenter, double-blind study [124]. Another systematic review, which included 35 studies,

associated St. John's wort with more treatment responders and fewer adverse effects than placebo and standard antidepressant interventions (including psychotherapy and antidepressant medication) [125].

2.5.5 In pregnancy

Safety

St. John's wort is one of the most frequently used herbal medicines in pregnancy [96]. However, the results of animal studies, most of which have methodological limitations, are inconclusive [126], and the potential risk to pregnant women is still unclear, as only a few studies are available [127, 128]. In an *in vivo* study with 54 rats, there was evidence of a reduced number of offspring with St. John's wort exposure prior to conception, and prolonged duration of pregnancy and worsened fetal development (reduced body weight) with exposure during pregnancy. Therefore, they do not recommend the intake of St. John's wort to women who want to become pregnant or are pregnant, as it may be harmful to the fetal and maternal health [129]. An *in vitro* study showed that hyperforin inhibited cell proliferation and differentiation in mouse embryonic stem cells [33]. An *in vitro* study showed that hyperforin at typical doses has only a low risk of embryotoxicity, but when ingested in large amounts can result in the inhibition of cell proliferation and differentiation in mouse embryonic stem cells, which may pose embryotoxic and teratogenic risks to pregnant women [130]. A review from 2014 [95] assessed St. John's wort as well tolerated and safe in pregnancy, although the data are based on only one case report [131] and one prospective, observational study [128]. A recent study of St. John's wort-exposed pregnancies which is based on claims data showed that the administration mainly occurred in the first trimester in 420/496 (84.7%) of pregnancies. However, unlike other antidepressants, it was used as an alternative in only a minority. Overall, 11% of pregnancies ended in non-live births and major malformations were noted in 18/312 babies (5.8%), 17 of whom were St. John's wort exposed in the first trimester. The relative risk of major malformations in babies exposed to St. John's wort during the first trimester compared with those exposed only during the second or third trimester was 3.56 (0.48-26.17). The relatively high rates of non-live births and malformations after first trimester exposure to St. John's wort should be interpreted with caution, yet the results are conspicuous [132].

Recommendations for clinical practice

European Medicines Agency

The HMPC of the EMA does not recommend the use of St. John's wort during pregnancy and lactation due to equivocal results in animal studies and insufficient clinical data [133, 134].

Embryotox

According to the Institute of Clinical Pharmacology and Toxicology at the Hospital Charité in Berlin, St. John's wort is considered a "drug" with conflicting or insufficient study data. Therefore, they recommend that the use of St. John's wort for depressive disorders is acceptable during pregnancy, if interactions with comedications (cave: induction of P-gp and CYPs [119]) are reviewed. The implications of its use in pregnancy are careful pregnancy monitoring and close psychiatric contacts to counteract crises in the mother and developmental complications in the fetus (preterm birth aspirations, growth retardation) in a timely manner. They suggest sertraline or citalopram as more appropriate alternatives [135].

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Data in humans to date do not indicate a significantly increased risk of teratogenicity, but the data are not extensive enough to make a firm statement. Therefore, even in the absence of interacting comedication, use in pregnancy should only be undertaken after consideration of various alternatives [136].

2.6 California poppy

California poppy (*Eschscholzia californica* Cham., **Figure 8**) belongs to the poppy family of Papaveraceae, and is also called Goldmohn or Schlafmützchen in German. The plant is a 30-60 cm tall annual to perennial herb with sparse fern-like blue-green foliage. The four-petaled silky-textured flowers, solitary on long stems, are pale yellow to orange in color with numerous yellow stamens. California poppy is native in the United States of America (USA; from California to New Mexico) and is mainly grown in the south of France. An ornamental plant exists in Europe since the mid-19th century which is a hybrid of several subspecies/varieties of the species *Eschscholzia californica* [137].

Figure 8. California poppy (*Eschscholzia californica* Cham.)
Photo source: Sodel Vladyslav/Shutterstock [138]

2.6.1 Historical use

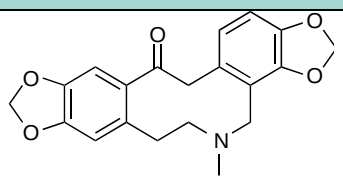
California poppy was first described by Adelbert von Chamisso, a German botanist, in 1820. Chamisso was participating in a scientific expedition from 1815-1818 and was a naturalist on the Russian ship Rurik, which was led by Otto von Kotzebue. The Rurik landed in San Francisco in October, and the California poppy was among the few plants still in bloom. Chamisso named the genus in honor of his friend Johann Friedrich Eschscholtz, the physician and naturalist who also took part in the expedition [139]. As a result, the flower is now known as the state flower of California and is therefore depicted on numerous welcome or street signs. Traditionally, California poppy was used by the Native American population in the western USA as a sedative and analgesic. According to the Handbook of Medicinal Herbs, California poppy is reported to have analgesic, antispasmodic, anxiolytic, calmative, diaphoretic, diuretic, emetic, lactifuge, narcotic, piscicide, poison, sedative, soporific, and uterotonic activities [140]. Today the herb is mostly used and known for its sedative and analgetic properties. The herbal medicine has been available as a powdered dry herb in hard capsules on the European market (France and Spain) since 1982, hence its traditional use in Europe [141]. In Switzerland, the medicinal drug can be obtained in pharmacies

and drugstores as capsules containing the powdered herb (Arkocaps® Escholtzia, Arko Diffusion S.A., Geneva) [61].

2.6.2 Phytochemistry

The chemical composition of the California poppy is well known. The entire plant contains alkaloids, although the composition varies considerably in the different organs (e.g., aerial parts, roots, stems, leaves, flowers, capsule valves, seeds). The roots are richer with 2.7% alkaloids in contrast to reported 0.29-0.38% alkaloids in the herb [137]. Six different groups of alkaloids have been described so far including the pavine alkaloids (e.g. californidine, escholtzine) which are most abundant and characteristic of this genus; protopine alkaloids (e.g. protopine **(Table *5*)**, cryptopine, α-allacryptopine); aporphine alkaloids (e.g. N-methyllaurotetanine, glaucine); benzophenanthridine alkaloids; benzylisoquinoline alkaloids; and tetrahydroprotoberberine alkaloids [141]. According to Guédon et al., the alkaloids in the aerial parts of the plant consist of 65.0% californidine, 18.5% escholtzine, and 6.3% protopine [142].

Table 5. Physicochemical characteristics of protopine [111].

Study compounds	Protopine
Chemical structure	
Empirical formula	C_{20} H_{19} NO_5
Molecular weight (g/mol)	353.37
Hydrogen-Donors	0
Hydrogen-Acceptors	6
Rotatable bonds	0
Rings	5
Lipinski violations	0
Solubility in water (mg/mL, 25°C)	46.5 (soluble)
Partition coefficient log $K_{oc/w}$	2.91 (optimal)

2.6.3 Pharmacological activities

California poppy extracts have shown sedative and anxiolytic effects *in vivo* [143, 144], and these properties have been traditionally attributed to the isoquinoline alkaloids (such as californidine and eschscholzine) [141]. The aqueous alcohol extract of California poppy showed affinity for GABA receptors in mice, explaining the sedative and anxiolytic effects. In addition, the extract also elicited

peripheral analytic effects but did not possess antidepressant, neuroleptic, or antihistaminic effects [145]. Several *in vitro* studies suggest that the plant contains several central nervous system (CNS)-active compounds. Protopine and allocryptopine were found to bind to $GABA_A$ receptors in rat synaptic membrane preparations [146, 147] and are also inhibitors of serotonin and noradrenaline transporters expressed in murine S6 and N1 cells, respectively [148]. In addition, they also act as anti-inflammatory agents and as acetyl-cholinesterase inhibitors both *in vitro* and *in vivo* [149]. The aporphine alkaloid N-methyllaurotetanine was shown to be a ligand at serotonin 5-HT_{1A} receptors expressed in human CHO cell membranes [150]. However, it must be noted that some of those pharmacological effects only occur at a certain amount of alkaloids (micromolar range). However, the total amount of alkaloids from a conventional OTC preparation (300 mg) would be too low (submicromolar range) to cause these desired biological effects *in vitro* and *in vivo* [151]. In addition, the alcoholic extract of California poppy and its major alkaloids (protopine, escholtzine, allocryptopine, and californidine) have the potential to inhibit the metabolism of various CYPs, which may result in diverse drug-drug interactions [152]).

2.6.4 Clinical evidence in the treatment of mild mental disorders

The plausibility of the efficacy is based only on long-standing use and experience, and there only are few clinical studies available. In an open-label study, 60 patients (38 women, 20 men, 2 children) suffering from sleep disorders were treated with California poppy for 6 months. Subsequently, 43% of patients reported excellent results (improved sleep quality); 43% of patients reported good results, while 14% discontinued the study due to lack of efficacy [141]. A fixed combination of California poppy and valerian was beneficial in the treatment of insomnia in an observational study in adults (increasing night sleep duration and sleep efficiency, decreasing the number of awakenings and anxiety score) [153]. The efficacy of California poppy together with hawthorn (*Crataegus oxyacantha*) and magnesium in treatment of anxiety disorders was investigated in a double-blind, randomized, placebo-controlled clinical trial. In a total of 264 patients, the above-mentioned plant combination (and magnesium) was safe and more effective than placebo in the treatment of mild to moderate anxiety disorders [144]. Another open-label study - including 20 patients with chronic pain - focused on the analgesic efficacy evidence, while also collecting information on the safety of the product. A questionnaire was also used to assess insomnia. A standardized California poppy extract was therefore administered once or twice daily for up to one month. The authors concluded that this plant extract can be used to treat chronic (as well as mild to moderate nocturnal) pain and as a mild sedative to treat pain-related insomnia [141]. A review from 2013 suggested California poppy for the treatment of anxiety disorders based on the available preclinical studies [154].

2.6.5 In pregnancy

Safety

There is no *in vitro* data on the genotoxicity, reproductive and developmental toxicity regarding the herbal extract and constituents thereof.

Recommendations for clinical practice

European Medicines Agency

The HMPC of the EMA does not recommend the use of California poppy during pregnancy and lactation due to the absence of sufficient data and not yet established safety profile [155].

Embryotox

No entries/recommendations available

Schweizerische Akademie für perinatale Pharmakologie

No entries/recommendations available

2.7 Valerian

Valerian (*Valeriana officinalis* L., **Figure 9**), also referred to as common valerian or garden valerian, is a perennial plant belonging to the family of Caprifoliaceae. The roots and rhizome of the plant are used as a drug. With pinnate leaves and sweet-smelling pink or white flowers, valerian grow between 30-150 cm tall, depending on the cultivar. The plant is native to Europe and Asia and has naturalized in northeastern America. Valerian is primarily sourced from crops in the Netherlands, Belgium, and Eastern Europe, but is nowadays increasingly cultivated in Germany [103].

Figure 9. Valerian (*Valeriana officinalis* L.)
Photo source: Snowbelle/Shutterstock [156]

2.7.1 Historical use

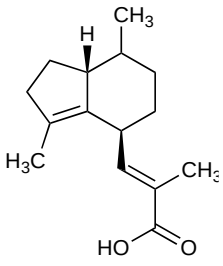
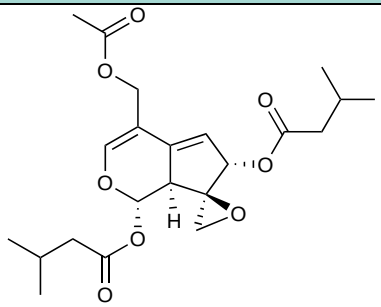
Valerian has a long history of medicinal use, dating back to the era of the Greek physicians Hippocrates (460-377 AD) and Dioscorides, who used the dry root as a remedy for liver diseases, urinary tract complications, and menstrual cramps. In Europe, the use of valerian as a monotherapy was first described by Hahnemann (1793-1799) for the treatment of epilepsy, various convulsions, worm diseases and hysterical complaints. Chamisso (1781-1831) also describes valerian root as antispasmodic, effective against worm diseases and as strengthening. Valerian was first mentioned in connection with sleeplessness in 1976, among many other effects such as antihysteric, aphrodisiac, diuretic, analgesic, antitussive, etc. [157]. Today, valerian is a traditional herbal medicinal product used to relieve mild symptoms of mental stress and to aid sleep [158].

2.7.2 Phytochemistry

Valerian root contains many different natural substances, the most important of which are [103]:

- **Mono- and sesquiterpenes:** the essential oil is composed of mono- and sesquiterpenes, with the content varying based to the origin (0.3-2.1%; according to the European Pharmacopoeia (Ph. Eur.) at least 0.3% for the cut drug). A total of about 160 components have been identified to date, with bornyl acetate being predominant, among others. Other important sesquiterpenes include valerianol, valeranone, α-kessylacetate, β-eudesmol, valerenal, tamariscene, and the pacifigorgianes.
- **Sesquiterpenic acids:** they are heavily volatile and expressed as valerenic acid **(Table *6*)**; according to Ph. Eur. not less than 0.10% in cut drug)
- **Valepotriates:** 0.1-0.2% with valtrate **(Table *6*)** and isovaltrate as main components. These are very unstable, and are thermo-, alkali- and acid-labile. In aqueous or ethanolic extracts only their degradation products occur which are, baldrinal, homobaldrinal, valeric and isovaleric acids, among others.
- **Lignans:** various, in small amounts (0.2%)
- **Free amino acids:** arginine, alaninie, GABA, and glutamine in small amounts
- **Flavonoids:** 8-methylapigenin, 2S-hesperidin, and linarin in small amounts

Table 6. Physicochemical characteristics of valerenic acid and valtrate [111].

Study compounds	Valerenic acid	Valtrate
Chemical structure		
Empirical formula	$C_{15}H_{22}O_2$	$C_{22}H_{30}O_8$
Molecular weight (g/mol)	234.33	422.47
Hydrogen-Donors	1	0
Hydrogen-Acceptors	2	8
Rotatable bonds	2	11
Rings	2	3
Lipinski violations	0	0
Solubility in water (mg/mL, 25°C)	0.25 (soluble)	0.8 (soluble)
Partition coefficient log $K_{oc/w}$	4.63 (lipophilic)	2.22 (optimal)

2.7.3 Pharmacological activities

Despite intensive efforts, it has not yet been possible to clearly identify the substances that determine the efficacy of valerian. Of the pharmacological studies, the following ones should be highlighted [82, 103]: Valerenic acids have muscle relaxant, spasmolytic, and CNS depressant effects since they inhibit the degradation of the inhibitory neurotransmitter GABA in the CNS [159]. The water-soluble lignans are discussed as the main active ingredient responsible for the sedative effect. The lignan olivil is a partial agonist of adenosine A1 receptors with a high affinity, thereby lowering the postsynaptic potential of neurons in the brain [160-162]. The agonistic activity at the adenosine A1 receptor is likely to be largely responsible for the sleep-inducing effect of alcohol-aqueous valerian extracts. Among the flavonoids, there are some candidates with activity on the CNS, such as 6-methylapigenin which is a benzodiazepine binding site ligand of the GABA receptor and was found to have anxiolytic properties [163], 2S-hesperidin and linarin which revealed sedative and sleep-enhancing activities (mechanism unknown) [164, 165]. However, due to their low bioavailability, flavones play most likely a minor role in the sedative component. Nevertheless is the effectiveness of valerian preparations less due to individual substances than to synergistic effects, i.e. to a combined action of all ingredients [103].

2.7.4 Clinical evidence in the treatment of mild mental disorders

The following effects of valerian preparations have been proven by a growing number of clinical studies: Improvement of sleep quality [166, 167] with decreasing the amount of time it takes to fall asleep, reduction of nocturnal awakenings [168-170]; improvement in daytime well-being (after 2-3 weeks of therapy) [82]; decreased central hyperactivity [82]; and reduction of anxiety (see [171] for further references).

A seven week treatment of a valerian and lemon balm combination resulted in enhancement of concentration, hyperactivity, and impulsiveness in an observational study in primary school children [172]. Additional clinical trials have been conducted on a combination of a valerian and hops extract where a randomized, placebo-controlled trial against diphenhydramine [173] and a randomized, placebo-controlled double-blind trial [174] conclude that the valerian-hops combination has a modest hypnotic effect compared to placebo and significantly reduces sleep latency, respectively. The valerian-hops combination also resulted in sleep-inducing effects in a randomized, placebo-controlled sleep-electroencephalogram (EEG) study [175].

In pregnancy

Safety

Valerian preparations are among the most commonly used medications during pregnancy [176] as shown in a data analysis from the Swedish Birth Register from 1995 to 2001 (n=860'215 women). No adverse effects on pregnancy outcome have been reported [177]. *In vivo*, there were conflicting results: On one hand, it was reported that chromosomal aberrations, spermatozoa abnormalities, and depletion of nucleic acids in testicular cells occurred in mice after 7 days of oral administration of valerian [178]. Moreover, the offspring of female mice treated with high doses (1.2g/kg) of valerian extract had lower levels of zinc in the fetal brain, while the other parameters (weight, volume of cerebral cortex, copper brain tissue content) remained unchanged [179]. On the other hand, treatment of rats between the 8th and 15th day of gestation with ethanolic valerian extract did not cause abnormalities in either dams or their offspring [180].

Recommendations for clinical practice

European Medicines Agency

The HMPC of the EMA does not recommend the use of valerian during pregnancy and lactation due to the absence of sufficient data and not yet established safety profile [158].

Embryotox

The Institute of Clinical Pharmacology and Toxicology (at the Hospital Charité in Berlin) classifies valerian as a "drug" for which there are conflicting or still insufficient study data. Therefore, they recommend that the use of valerian for restlessness and nervousness-induced insomnia is acceptable during pregnancy. However, attention should be paid to the use of non-alcoholic preparations. For a short-time therapy of sleep disorders they suggest diphenhydramin or doxylamin as more appropriate alternatives [181].

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Practical experience, including from surveys on the use of herbal products in pregnancy, speaks in favor of the use in pregnancy, where so far no adverse effect of valerian preparations on mother and child could be found. Aqueous extracts (as ready-to-use medicinal products or as tea) of valerian root are generally suitable for daytime sedation or sleep induction (onset of action usually after one month); ethanolic extracts are suitable as daytime sedatives for anxiety states. The dosage instructions should be followed [182].

2.8 Lavender

Lavender (*Lavandula angustifolia* Mill., **Figure *10***) belongs to the family of Lamiaceae and is also known as English lavender, garden lavender or true lavender [183]. It grows to a subshrub (approximately 0.5-1 m tall) with narrow, lance-shaped leaves. The deep blue-grey flowers are stripped from the long stem just before blossoming, dried and thus can be used as a drug. The plant is native to the Mediterranean region where it is also cultivated in a larger scale and imports of the drug are mainly from France, Spain, and Eastern Europe. Lavender has an intense, pleasant aromatic-scented odor, which is why it is found not only as a phytopharmaceutical but also as a massage and bath oil or in scented soaps [103]. The botanical name comes from the Latin word *lavare* (to wash) and refers to the time of the Romans, who used the lavender flowers as refreshing bath additive. *Angustifolia* derives from the *angustus* (Latin for small) and refers to the narrow leaves of the plant [184].

Figure 10. Lavender (*Lavandula angustifolia* Mill.*)*
Photo source: Dionisvera/Shutterstock [185]

2.8.1 Historical use

The earliest writings mentioning lavender are by the Greek scholar Theophrastus (370- 285 BC), and it is known that in ancient Greece, Persia, and Rome lavender was used as a perfume for baths and linens and an antiseptic. The ancient Egyptians made mummification casts by soaking linen in asphalted lavender oil. However, lavender did not become an important medicinal plant until around 77 AD when Dioscorides discovered its presence, describing laxative and refreshing properties. The Roman physician and philosopher Galen (129-199 AD) used lavender as an antidote for poisoning and insect bites. In the writings of Pliny the Elder, the Roman naturalist and philosopher, lavender was used to treat sadness, among other things. In the 11th century, Benedictine monks brought lavender across the Alps to the monastery gardens of northern Europe, and thus the first mention of the species *Lavandula angustifolia* comes from Hildegard of Bingen. In Tibetan Buddhist medicine, lavender is used to treat insanity and psychosis [183, 186].

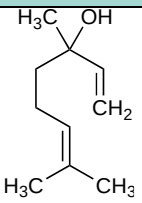
Overall, lavender essential oil has a long history of folk and traditional therapeutic use, so it is also credited with numerous properties: antibacterial, antidepressant, antifungal, antispasmodic, carminative (promotes the elimination of flatulence), wound-healing, and balancing and calming, with the latter playing a major role today [187]. Today, lavender is widely used in Europe and America as an anxiolytic and sleep aid. The EMA also lists "relief of mild symptoms of mental stress and exhaustion" as an indication in the Community Herbal Monograph of lavender [188].

2.8.2 Phytochemistry

Lavandula angustifolia Mill. is composed of more than 100 constituents, the most important of which are [103]:

- 1-3% **essential oil** (*Lavandulae aetheroleum*): main components are R-(-)-linalyl acetate (30-50 %; Ph. Eur.: 25.0-46.0%) and R-(-)-linalool (20-45%; Ph. Eur.: 20.0-45.0%, **Table 7**) in varying proportions, besides (Z)-ß-ocimene, terpinene-4-01, ß-caryophyllene, lavandulyl acetate, 1,8-cineole and other monoterpenes. A Gas Chromatography/Mass Spectrometry (GC/MS) analysis revealed that lavender oil contains 26 constituents, of which linalool (26.1%) and linalyl acetate (26.3%) are the most abundant [189]
- 2-3% **lamiaceous tannins**: especially chlorogenic acid and rosmarinic acid, phenolic carboxylic acids
- 0.35% **flavonoids**
- Traces of **phytosterols** and **triterpenes**

Table 7. Physicochemical characteristics of linalool [111].

Study compounds	Linalool
Chemical structure	
Empirical formula	$C_{10}H_{18}O$
Molecular weight (g/mol)	154.25
Hydrogen-Donors	1
Hydrogen-Acceptors	1
Rotatable bonds	4
Rings	0
Lipinski violations	0
Solubility in water (mg/mL, 25°C)	0.17 (soluble)
Partition coefficient log $K_{oc/w}$	3.29 (optimal)

2.8.3 Pharmacological activities

Lavender essential oil is known for a wide range of biological effects [190, 191], the most important of which are the anxiolytic and antidepressant effects, and it is very active at low concentrations in several rodent behavioral models typically used to test the latter two properties [192-195]. In animal studies, linalool has been identified as the main pharmacologically active constituent involved in the antianxiety effects of lavender oil [189]. In addition, linalool has been shown to have neurochemical effects in mice due to a dose-dependent inhibition of glutamate (primary excitatory neurotransmitter in the CNS) binding, and it has been suggested that a potentiation of the neurotransmitter GABA may lead to the hypnotic and anticonvulsant effects of lavender [196]. However, it is important to note that many receptor systems (GABAergic and glutamatergic) as well as several neurotransmitter transporters were only affected by unrealistically high concentrations of lavender oil which are not in agreement with the low effective doses, plasma and tissue levels of lavender oil [197, 198]. Therefore, data on the possible mechanism of action are still inconclusive and often controversial. In a certain analogy to the anxiolytic pregabalin, lavender oil was found to have a moderate inhibition of presynaptic voltage dependent calcium channels (VDCC), but without serving the same target as pregabalin (lavender oil: mainly T-type and N-type channels and to some extent P/Q-type channels, pregabalin: mainly P/Q-type channels) [192]. In great analogy to classical antidepressants, lavender oil improves several aspects of neuroplasticity, through activation of intracellular signaling kinases such as protein kinase A (PKA) and mitogen-activated protein kinase (MAPK), which in turn leads to activation of the transcription factor cAMP response element-binding protein (CREB) [197, 199].

2.8.4 Clinical evidence in the treatment of mild mental disorders

Lavender has been shown to be effective in the treatment of anxiety, according to a recently published systematic review [200]. The anxiolytic effect was superior to placebo [201] and comparable to that of a benzodiazepine (low-dosed lorazepam [202] and the SSRI paroxetine [203]) in adults with generalized anxiety disorder, and fewer adverse effects were reported than with the synthetic drugs. Another randomized, double-blind trial in patients with anxiety-related restlessness and disturbed sleep confirmed the calming and anxiolytic efficacy of lavender essential oil [204]. Furthermore, a double-blind, randomized study with 212 patients observed that lavender exerts a secondary sleep-promoting effect through its anxiolytic action, which is not per se mediated by sedation [205]. Hence several clinical trials proved good effectiveness and tolerability of lavender as a phytopharmaceutical, predominantly in the treatment of anxiety. In addition, lavender tea has also been shown to have a positive therapeutic effect on depressed patients, as the effects of the antidepressant citalopram was enhanced in a study including 80 patients with major depression

[206]. In a randomized placebo-controlled trial, lavender cream was effective in reducing anxiety, stress and depression [207].

2.8.5 In pregnancy

Safety

In a double-blind randomized placebo-controlled trial with 141 pregnant women at 25 to 28 week gestation, the effectiveness of lavender cream with and without footbath was investigated, however, without focusing on the safety [207]. In aromatherapy, lavender essence is safe and effective for pain reduction after cesarean section [208] or episiotomy [209]. Consumption is often labeled as contraindicated during pregnancy due to its emmenagogue (stimulating blood flow) potential [93, 210].

Recommendations for clinical practice

European Medicines Agency

The HMPC of the EMA does not recommend the use of lavender during pregnancy and lactation due to the absence of sufficient data and not yet established safety profile [188].

Embryotox

No entries/recommendations available

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No entries/recommendations available

2.9 Hops

Hops (*Humulus lupulus* L., Cannabaceae, **Figure *11***) belongs to the Cannabaceae family of flowering plants. The dioecious, perennial, herbaceous climbing plant grows 3-6 m high, in cultivation up to over 10 m, and is grown in many countries of the temperate climate zone. The drugs consist of the dried female cone-shaped fruits (hop cones) or of their glands. The hop cones are greenish-yellow in color, have a strong spicy odor and a somewhat bitter taste. They are surrounded by partially overlapping scales (bracts and bracteoles) and reach maturity at the end of summer or beginning of autumn where they can be harvested. Inside these scales are the oil glands, which form an orange-yellow resinous powder which constitute lupulin. It also tastes bitter and contains numerous compounds that are of great economic interest, especially for the beer brewing industry. For this reason, only female hop plants are being cultivated [103, 211]. The origin of the genus name *Humulus* is in the Latinized version of 'chmele' (Slavic language for hop). The species name *lupulus*, on the other hand, is a diminutive of lupus (Latin for wolf) and is based on the strangulating growth of the wild plant in behavior towards other plants, which has been compared to the destructive behavior of wolves towards sheep The common name hops is derived from the Anglo-Saxon 'hoppan' (to climb) [212].

Figure 11. Hops (*Humulus lupulus* L.)
Photo source: New Africa/Shutterstock [213]

2.9.1 Historical use

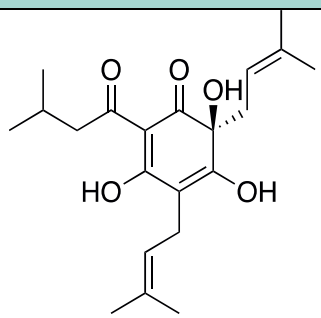
Hops was first used already around 1000 AD for brewing purposes whereby originally because of its antimicrobial effect as a preservative and only later to give the beer a bitter taste [214]. Only much later did the plant also become appreciated for its medicinal properties. Paracelsus used hops as a digestive aid, and Matthiolus (1501-1577), an Italian physician, medical writer, and botanist, mentioned its diuretic and bile-promoting effects. At the same time, young hop shoots were also used to purify blood, liver, and spleen. In 1814, strongly tonic properties as a bittering agent (amarum) and calming properties were attributed to hop flowers [212]. Hops have only been used as a sedative and sleep aid since the 19th century, when it was observed that hop pickers tired easily,

presumably because the hop resin from their hands accidentally transferred to their mouths [215]. In 1953, it was shown that hops contain an equivalent of 20-300 μg estradiol/g, as a folk legend reported menstrual-stimulant properties of hops. Later in 1973, it is claimed that hops can be also used to treat various gynecological diseases [216]. Nowadays, the HMPC of the EMA reports the traditional use of hops to relieve mild symptoms of mental stress and to aid sleep [217]. According to the German Commission E Monograph [218] hops can be used to treat "mood disturbances such as restlessness and anxiety, sleep disturbances". It is also externally used for the treatment of ulcers and skin injuries. In Bavaria, the hop cushion is used for calming and for sleep disorders [103].

2.9.2 Phytochemistry

The bitter acids, volatile essential oil, and the polyphenols are the most important constituents of hops [211]. The bitter principles consist mainly of phloroglucinol derivatives which are found in hop cones (15-30%) and hop glands (50-80%) and are known as α-acids (humulones, **Table *8***) and β-acids (lupulones). Analytical data reported a content of 2-12% of α-acids in hops cones, with humulone as the major compound (35% to 70%). 2-Methyl-3-buten-2-ol is a degradation product of these bitter acids and is therefore found only in hop cones that have been stored for a longer period, with a maximum content of about 0.15% of the dry weight [103, 214, 215, 219, 220].

Table 8. Physicochemical characteristics of humulone [111].

Study compounds	Humulone
Chemical structure	
Empirical formula	$C_{21}H_{30}O_5$
Molecular weight (g/mol)	362.46
Hydrogen-Donors	3
Hydrogen-Acceptors	5
Rotatable bonds	7
Rings	1
Lipinski violations	0
Solubility in water (mg/mL, 25°C)	2.72 (soluble)
Partition coefficient log $K_{oc/w}$	3.96 (optimal)

The hop cones (0.3-1%) and hop glands (1-3%) also consist of essential oil, which is mainly composed of monoterpenes (e.g., myrcene) and sesquiterpenes (e.g., humulene, caryophyllene). Polyphenols include flavonoids (0.5-1.5%) with flavonol glycosides (e.g., kaempferol, quercetin, quercitrin, rutin) and prenylated chalcone flavonoids (e.g., xanthohumol, isoxanthohumol) [103, 214, 215, 219, 220].

2.9.3 Pharmacological activities

Considering the effects of the three main groups of secondary metabolites of hops (resins, essential oils, and polyphenols), it becomes evident that they exhibit a wide range of pharmacologically important properties [219, 221, 222]: In addition to antioxidant, anti-inflammatory, and anticancer properties, 8-prenylnaringenin (a prenylflavonoid), found almost exclusively in hops, is considered the most potent phytoestrogen identified so far [223]. The plant is also increasingly mentioned in connection with the metabolic syndrome, as some constituents have obesity- and diabetes-related activities. For example, xanthohumol and iso-α-acids can improve health by affecting lipid metabolism, glucose tolerance, and body weight, which is promising in terms of metabolic syndrome treatment [224-226].

Various hop constituents such as essential oils or hop resins can modulate specific neurotransmitter receptors which is the underlying principle of their sedative, tranquilizing or other neuropharmacological effects:

Hops extracts reportedly activate melatonin receptors *in vivo* [227, 228] and binding affinities to melatonin and serotonin receptors could be as well demonstrated *in vitro* [229], which may explain, at least in part, the sleep-inducing properties. Zanoli et al. studied the properties of α- and β-bitter acids and showed that they, as well as commercial extracts used for beer production, affect the CNS of rats [230] and humulone was found to act as a positive allosteric modulator of recombinant $GABA_A$ receptors expressed in HEK-293 cells [231].

2.9.4 Clinical evidence in the treatment of mild mental disorders

Various phytomedicines and food supplements containing hops are available in the EU [232] and in Switzerland. However, in Switzerland these products always contain additional plant extracts such as valerian, lemon balm and/or passionflower, and the content in bitter acids is not specified [61]. Until recently, preparations consisting of a fixed valerian-hops combination represented the most frequently administered form of plant-based sleeping agents and sedatives [167, 175]. The clinical support for the efficacy of hops as a sedative is therefore only based on studies with the mentioned combination with valerian (Ze 91019): Schellenberg et al. showed that Ze 91019 has an influence on the CNS within one hour after administration, which was recorded by EEG responses

in 16 healthy volunteers. The patients showed an increase in alpha-1 power, which is a wavelength that indicates relaxation. This also reduced beta-2 power, which indicates increased alertness and mental activity [161]. In a randomized, placebo-controlled, parallel-group clinical trial involving 184 enrolled subjects with mild insomnia, Ze 91019 (and diphenhydramine) was shown to be safe and did not cause recurrence of insomnia when the medication was discontinued. In addition, improvement in sleep was associated with improved quality of life [173]. In another randomized, double-blind, placebo-controlled, parallel-group study, 30 patients with non-organic insomnia were administered either Ze 91019, placebo, or valerian alone for 4 weeks. Polysomnographic recordings showed that sleep latency (time between turning off the light and reaching sleep phase 2) was significantly reduced by the addition of hops. In terms of safety, there were no adverse events in any of the three groups [174].

2.9.5 In pregnancy

Safety

No clinical or *in vivo* data are available on the safety of hops preparations in pregnancy.

Recommendations for clinical practice

European Medicines Agency

The HMPC of the EMA does not recommend the use of hops during pregnancy and lactation due to the absence of sufficient data, not yet established safety profile, and no fertility data available [217].

Embryotox

No entries/recommendations available

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No entries/recommendations available

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4 RESULTS AND DISCUSSION

This dissertation is based on five peer-reviewed publications:

Chapter 1

Use of herbal medicines for the treatment of mild mental disorders and/or symptoms during pregnancy: A cross-sectional survey

Giulia Gantner, Deborah Spiess, Eliane Randecker, Katharina C. Quack Lötscher, Ana Paula Simões-Wüst

Chapter 2

Medicinal plants for the treatment of mental diseases in pregnancy: An *in vitro* safety assessment

Deborah Spiess, Moritz Winker, Antoine Chauveau, Vanessa Fabienne Abegg, Olivier Potterat, Matthias Hamburger, Carsten Gründemann, Ana Paula Simões-Wüst

Chapter 3

Advanced *in vitro* safety assessment of herbal medicines for the treatment of non-psychotic mental disorders in pregnancy

Deborah Spiess, Moritz Winker, Alexandra Dolder Behna, Carsten Gründemann, Ana Paula Simões-Wüst

Chapter 4

Placental passage of protopine in an *ex vivo* human perfusion system

Deborah Spiess, Vanessa Fabienne Abegg, Antoine Chauveau, Andrea Treyer, Michael Reinehr, Mouhssin Oufir, Elisa Duong, Olivier Potterat, Matthias Hamburger, Ana Paula Simões-Wüst

Chapter 5

Transplacental passage of hyperforin, hypericin, and valerenic acid

Deborah Spiess, Vanessa Fabienne Abegg, Antoine Chauveau, Joshua Rath, Andrea Treyer, Michael Reinehr, Sabrina Kuoni, Mouhssin Oufir, Olivier Potterat, Matthias Hamburger, Ana Paula Simões-Wüst

Chapter 1

Chapter 1

Use of herbal medicines for the treatment of mild mental disorders and/or symptoms during pregnancy: A cross-sectional survey

Giulia Gantner, **Deborah Spiess**, Eliane Randecker, Katharina C. Quack Lötscher, Ana Paula Simões-Wüst

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Little is known about the treatment of mild mental disorders and/or symptoms (MDS) during pregnancy. Our main purpose was to compare the use of herbal medicines during pregnancy in women with and without MDS. A questionnaire consisting of 21 multiple-choice questions was distributed in the participating obstetrics clinics or birth centers in the Canton of Zurich, in Switzerland, from August 2018 to March 2019; 398 questionnaires were considered in the analysis. The use of any type of herbal medicines - including pharmaceutical herbal products as well as teas - during pregnancy was reported by 358 women (out of 398, 89.9%). Of these, 272 participants used pharmaceutical herbal products, whereby ginger (49.2%), raspberry leaf (42.7%), bryophyllum (37.8%), chamomile (27.2%), lavender (22%) and iron-rich herbs (12.3%) were the ones most commonly mentioned. More than half (207/398, 52.0%) of all participants reported suffering from MDS during pregnancy; only a few took (synthetic) psychoactive medications (5/398, 1.3%). The prevalence of MDS was higher among users of pharmaceutical herbal products than among non-users (59.6% vs. 34.0%; p=0.001). Specific questions on candidate herbal medicines for the treatment of mild MDS revealed that bryophyllum (mentioned by 107 women), lavender (56 women) and valerian (20 women) were used to reduce stress, restlessness, sleep disorders and others, in part with perceived good to very good effectiveness and tolerability. The large majority of the pregnant women participating in the survey make use of herbal medicines. The particularly high prevalence of MDS among herbal medicine-users and the very rare use of synthetic psychoactive medications suggest that pregnant women rely on herbal medicines for treatment of mild MDS. The reported good effectiveness and tolerability of a few candidate herbal medicines deserve particular attention.

Contributions of Deborah Spiess to this publication: support in questionnaire design, verification of analyses and interpretation of data, data visualization, and manuscript revision

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Use of Herbal Medicines for the Treatment of Mild Mental Disorders and/or Symptoms During Pregnancy: A Cross-Sectional Survey

*Giulia Gantner, Deborah Spiess, Eliane Randecker, Katharina C. Quack Lötscher and Ana Paula Simões-Wüst**

Department of Obstetrics, University Hospital Zurich, University of Zurich, Zurich, Switzerland

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****Correspondence:***
Ana Paula Simões-Wüst
anapaula.simoes-wuest@usz.ch

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Little is known about the treatment of mild mental disorders and/or symptoms (MDS) during pregnancy. Our main purpose was to compare the use of herbal medicines during pregnancy in women with and without MDS. A questionnaire consisting of 21 multiple-choice questions was distributed in the participating obstetrics clinics or birth centers in the Canton of Zurich, in Switzerland, from August 2018 to March 2019; 398 questionnaires were considered in the analysis. The use of any type of herbal medicines–including pharmaceutical herbal products as well as teas–during pregnancy was reported by 358 women (out of 398, 89.9%). Of these, 272 participants used pharmaceutical herbal products, whereby ginger (49.2%), raspberry leaf (42.7%), bryophyllum (37.8%), chamomile (27.2%), lavender (22%) and iron-rich herbs (12.3%) were the ones most commonly mentioned. More than half (207/398, 52.0%) of all participants reported suffering from MDS during pregnancy; only a few took (synthetic) psychoactive medications (5/398, 1.3%). The percentage of use of pharmaceutical herbal medicines was higher among women reporting MDS than among the remaining women (90.0 vs 75.9%; $p < 0.001$). At the same time, the prevalence of MDS was higher among users of pharmaceutical herbal products than among non-users (59.6 vs 34.0%; $p = 0.001$). Specific questions on candidate herbal medicines for the treatment of mild MDS revealed that bryophyllum (mentioned by 107 women), lavender (56 women) and valerian (20 women) were used to reduce stress, restlessness, sleep disorders and others, in part with perceived good to very good effectiveness and tolerability. The large majority of the pregnant women participating in the survey make use of herbal medicines. The particularly high prevalence of MDS among herbal medicine-users and the very rare use of synthetic psychoactive medications suggest that pregnant women rely on herbal medicines for treatment of mild MDS. The reported good effectiveness and tolerability of a few candidate herbal medicines deserve particular attention.

Keywords: pregnancy, herbal medicines, phytopharmacy, mental health disorders, survey, bryophyllum, lavender, valerian

INTRODUCTION

Little is known about the treatment of mild mental disorders and/or symptoms (MDS) during pregnancy. Most medications for MDS may not only cause side-effects in the mother, but also easily cross the placental barrier and reach the foetus. Concerns on tolerability, teratogenicity and impact on neonatal outcomes exist (Sivojelezova et al., 2005; Rahimi et al., 2006; Grigoriadis et al., 2014; Yonkers et al., 2017; Gao et al., 2018). Pregnant women in need of such medications therefore face a dilemma between using and refraining from synthetic medications.

It is therefore understandable that a considerable proportion of women suffering from mild MDS opt for treatment with herbal medicines, which tend to be perceived as safe (Kalder et al., 2011; Pallivalappila et al., 2013). For instance in Germany, approximately one-fifth of the pregnant women who take herbal medications do it for psychological problems (Munstedt et al., 2013). Even though the toxicity of herbal medications taken during pregnancy has not in most cases been thoroughly investigated, a considerable proportion of health care professionals who deal with pregnant women–midwives, obstetricians, anaesthetists, and especially those with their own experiences–recommend herbal medicine (Stewart et al., 2014). In Switzerland, 40.6% of pregnant women reported using herbal medicine during pregnancy, which was higher than the average proportion detected in a multinational study [average 28.0% (Kennedy et al., 2013)].

Our main purpose was to compare the use of herbal medicines in women with and without MDS. Further goals were to characterise the use, perceived effectiveness and tolerability of a few candidate herbal medicines for mild MDS treatment.

METHODS

Study Design

The present analysis is based on self-reported data from obstetric patients participating in a cross-sectional survey undertaken between August 2018 and March 2019.

Ethics Statement

The study was conducted in accordance with the Helsinki Declaration and with Swiss laws and regulations. In compliance with Swiss Federal Law on data protection (Human Research Act, Article 2), since the data were anonymously collected, no special authorisation was needed. This was confirmed by jurisdictional declaration of the ethics committee of Zurich (Enquire BASEC-Nr. Req-2017-00966; letter from December 14, 2017).

Selection and Description of Participants

The survey took place in the Canton of Zurich–often considered to be representative of the Swiss population as a whole–whose inhabitants correspond to one-sixth of the entire Swiss population. Eight obstetric clinics and birth centres agreed to participate. In these institutions, pregnant women (at or after 28 weeks of pregnancy) or women in the puerperium were invited to participate in the survey if they had not previously completed the questionnaire, could read German, English, French or Italian, and were not emergency patients.

The aim of our survey was to compare the use of herbal medicines during pregnancy in women with and without MDS. Assuming that twice as many pregnant women take herbal medicines for symptoms related to physical conditions than for symptoms related to mental diseases [40 and 20%, respectively, compare with Munstedt et al. (2013)], a sample size of 106 in each group would allow the detection of a difference

TABLE 1 | Herbs mentioned in the questionnaire by common names and corresponding full taxonomical names.

Common names	Full taxonomic names
Mentioned under pharmaceutic products	
Ginger	*Zingiber officinale* Roscoe
Raspberry leaf	*Rubus idaeus* L
Bryophyllum/Goethe plant[a]	*Kalanchoe pinnata* (Lam.) Pers
Chamomile	*Matricaria chamomilla* L[b]
Lavender	*Lavandula angustifolia* Mill
Iron-rich herbs (Floradix®)[c]	-
Echinacea	*Echinacea angustifolia* DC
Lemon balm	*Melissa officinalis* L
Valerian	*Valeriana officinalis* L
St. John's wort	*Hypericum perforatum* L
Horsetail	*Equisetum arvense* L
Passionflower	*Passiflora incarnata* L
Hops	*Humulus lupulus* L
Horse chestnut	*Aesculus hippocastanum* L
Ginseng	*Panax ginseng* C.A. Mey[d]
Golden root (Vitango®)[e]	*Rhodiola rosea* L
California poppy	*Escholzia californica* Cham
Winter cherry	*Withania somnifera* (L) Dunal
Kava	*Piper methysticum* G. Forst
Mentioned under teas	
Fennel	*Foeniculum vulgare* Mill
Chamomile	*Matricaria chamomilla* L[b]
Raspberry leaf	*Rubus idaeus* L
Herbal mixture	-
Peppermint	*Mentha* × *piperita* L
Fruit mixture	-
Lime blossom	*Tilia* × *europaea* L
Verveine/verbena	*Verbena officinalis* L
Rosehip	*Rosa canina* L
Stinging nettle	*Urtica dioica* L
Lady's mantle	*Alchemilla alpina* L
Lemon balm	*Melissa officinalis* L
Orange blossom	*Citrus* × *aurantium* L
Aniseed	*Pimpinella anisum* L
Sage	*Salvia officinalis* L
Elderflower	*Sambucus nigra* L
Cumin	*Cuminum cyminum* L
St. John's wort	*Hypericum perforatum* L
Ginger	*Zingiber officinale* Roscoe
Horsetail	*Equisetum arvense* L
Valerian	*Valeriana officinalis* L

[a] *Bryophyllum was referred to also by an earlier genus name (that is also a trade name) and one common name, as common names (Goethe plant, life plant, air plant, love plant, and Cathedral bells) are relatively unknown in Switzerland.*
[b] *And/or* Chamaemelum nobile *(L.) All.*
[c] *Floradix® is a vitamin- and iron-containing food supplement with natural herbal extracts.*
[d] *And/or* Panax quinquefolius *L.*
[e] *Vitango® is a product based on Golden root (roots and rootstock).*

between the percentage of women taking herbal medicines in the groups with and without MDS. The survey was pursued until 106 women taking (pharmaceutical) herbal medicines in each of the groups with and without MDS had participated.

Questionnaire

The questionnaire consisted of 21 multiple-choice questions and was available in German, English, French and Italian. Piloting of the questionnaire–distributed to 14 women, at least three per language–was conducted to ensure readability and clarity of the questions.

Four of the 21 (complex) questions were related to the intake of herbal medicines during pregnancy and were inspired by a previous survey (Zuzak et al., 2009). The questionnaire distinguished between herbal infusions/teas and pharmaceutical herbal medicines, but did not specify the type of preparation. For a selection of six herbs, detailed information on perceived effectiveness and tolerability was collected. In the questionnaire, the common names of the herbs were used, with a few exceptions (see **Table 1**, also for the correspondence between the used names and full taxonomic names).

To contextualise our results, data on sociodemographic characteristics, acute/chronic disorders and symptoms and synthetic/conventional medications were collected [in part published elsewhere (Randecker et al., 2020)]. To avoid counting missing answers as "non-use" answers, the questions on medicinal herbal medicines included the option "never used"; in these cases, the number of total answers differs from question to question.

We defined an existing MDS on the basis of specific questions. If the women reported suffering from acute or chronic mental disorder, or related symptoms, or reported taking psychotropic drugs, they were considered to have MDS. No MDS severity assessment was performed; psychotic diseases were not addressed in the questionnaire.

Data Collection

A total of 1,653 envelopes–each one containing an information sheet, the questionnaire and a post-paid envelope addressed to the Department of Obstetrics, University Hospital Zurich–were handed out to potential participants in obstetric clinics and birth centres in the Canton of Zurich. Professionals were instructed by the study team to distribute the envelopes to patients during prenatal check-ups or during hospitalisation in the early puerperium. Sealing of the envelopes after insertion of the completed questionnaires was emphasised to the patients to ensure anonymity. Data were entered manually into a Microsoft Excel file.

Statistical Data Analysis

Descriptive statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), Version 25.0. for Windows (IBM® SPSS® Statistics). Pearson's chi-square test was used to compare use of herbal medicines between participants with and without MDS, and MDS prevalence between herbal medicines users and non-users. A two-sided p-value smaller than 0.05 was considered statistically significant; no correction for multiple testing was applied. The number of missing answers is depicted either in the tables or corresponding legends.

RESULTS

Number and Sociodemographic and Health-Related Characteristics of Participants

From a total of 1,653 questionnaires distributed, 398 were completed at or after 28 week's gestation or shortly after birth and returned either by post or through collection boxes placed in the various institutions (overall response rate: 398/1,653, 24.1%). The 398 participants were treated at the following institutions: University Hospital Zurich (n = 164), City Hospital Triemli (n = 61), Hospital Zollikerberg (n = 50), Hospital Bülach (n = 40), Paracelsus-Hospital Richterswil (n = 31), Hospital Limmattal (n = 21) and Delphys Birthing Center (n = 8); in some cases, other institutions (n = 10), more than one institution (n = 10) or none were named (n = 3).

Most women completed the questionnaires in German (362/398, 91.0%), followed by English (28/398, 7.0%), Italian (5/398, 1.3%) and French (3/398, 0.8%). The survey population was comprised mainly of women between 18 and 43 years, with a medium to high level of education (high school to university). The majority of women were from Switzerland (202/398, 50.8%), followed by Germany (64/398, 16.1%) and other European countries (86/398, 21.6%), America (19/398, 4.8%), Asia (16/398, 4.0%), Africa (2/398, 0.5%) and Australia (1/398, 0.3%), others (6/398, 1.5%). The majority of participants had delivered in the days before receiving the questionnaire (221/371, 59.6%); the remaining women were either in pregnancy weeks 28–37 (79/371, 21.3%) or 38-42 (71/371, 19.1%). Women were slightly more often primiparous (193/372, 51.9%) than multiparous. For detailed data, see **Supplementary Table S1**.

About a quarter of women reported chronic disorders (101/373, 27.1%), of which allergies (29/373, 7.8%), thyroid disorders (24/373, 6.4%), and headaches/migraines (20/373, 5.4%) were the most common (**Supplementary Table S2**). The following pregnancy-related acute disorders were the most common: 18.6% of women (69/371) reported suffering from gastroesophageal reflux, 17.0% (63/371) from iron deficiency/anemia, and 14.3% (53/371) from morning sickness. The most commonly reported symptoms during pregnancy were fatigue (294/370, 79.5%), nausea (248/365, 67.9%), heartburn (211/359, 58.8%) and shortness of breath (191/359, 53.2%). For additional information on disorders and symptoms during pregnancy, see **Supplementary Tables S2, S3**, respectively.

MDS During Pregnancy

Four percent of the women (15/372) reported one of the following chronic mental disorders: minor depression (n = 5); mood disorder (n = 5); anxiety disorder (n = 3); major depression (n = 1) and sleeping disorder (n = 1). Moreover, a prevalence of acute mental disorders during pregnancy of 13.2% (48/371) was observed, which

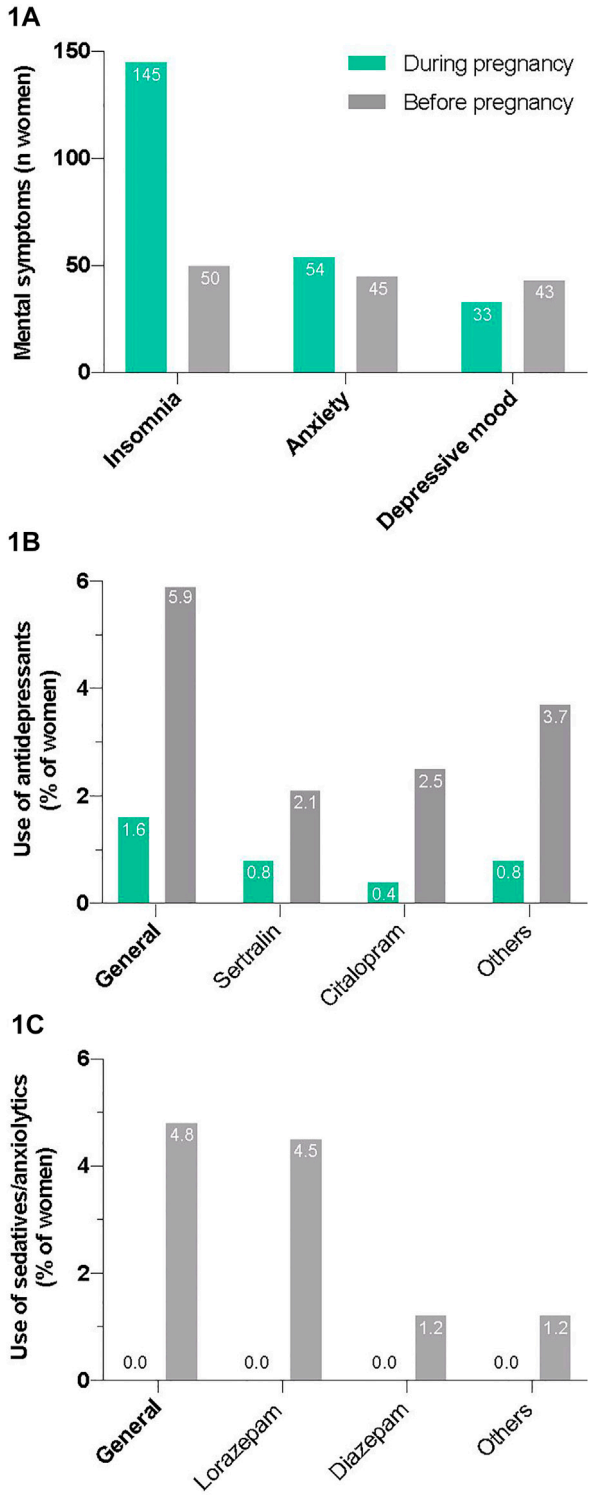

FIGURE 1 | Perceived mental symptoms **(A)** and reported use of antidepressants **(B)** and sedatives/anxiolytics **(C)** during pregnancy and in the time before.

included sleeping disorder ($n = 27$); mood disorder ($n = 13$); minor depression ($n = 5$) and anxiety disorder ($n = 3$) (**Supplementary Table S2**). More than half (51.3%) of the participants reported having suffered from a mental symptom during pregnancy (204/398), namely: insomnia (145/338, 42.9%), anxiety (57/320, 17.8%), and depressive mood (33/335, 9.9%). Compared with the pre-pregnancy period, the prevalence of anxiety and insomnia was higher, whereas the prevalence of depressive mood was slightly lower during pregnancy (**Supplementary Table S3**). **Figure 1A** provides an overview of the prevalence of mental symptoms during and before pregnancy.

Only a few participants used antidepressants (5/315, 1.6%) and none used sedatives/anxiolytics during pregnancy (**Figures 1B,C**; **Supplementary Table S4**). The number of participants taking antidepressants and sedatives/anxiolytics before becoming pregnant was markedly higher (19/321 or 5.9%; and 15/315 or 4.8%, respectively). Two women (2/240; 0.8%) answered that they were taking an antidepressant not mentioned in the questionnaire during pregnancy, namely escitalopram ($n = 1$) and paroxetine ($n = 1$), both selective serotonin reuptake inhibitors (SSRIs); additional other sedatives/anxiolytics were not mentioned.

Use of Herbal Medicines in General and Versus MDS

The vast majority of women reported using some type of herbal medications, either as teas or as pharmaceutic/clinical medicines, during pregnancy (358/398, 89.9%). Of these, 272 participants (272/325, 83.7%) used pharmaceutical herbal products, most frequently ginger (49.2%), raspberry leaf (42.7%), bryophyllum (37.8%), chamomile (27.2%), lavender (22.0%) and iron-rich herbs (12.3%); see **Table 2**. The majority of participants (263/358, 86.5%) who reported using herbal medicines during pregnancy, also used herbal medicines before the current pregnancy. The consumption of teas was very widespread in our study population (**Table 3**).

The percentage of use of pharmaceutical herbal medicines was higher among women reporting MDS than among the remaining women (teas not considered; 162/180, 90.0% vs 110/145, 75.9%; $p < 0.001$). Almost 60% of the women who used pharmaceutical herbal medicines suffered from MDS (162/272, 59.6%), which is significantly higher than the corresponding value for non-users (18/53, 34.0%; $p = 0.001$). More than half of the women who used any type of herbal preparation, teas and pharmaceutical herbal medicines, reported MDS (192/358, 53.6%). **Figure 2** shows the most frequently used herbal medicines, pharmaceutical herbal medicines and teas counted together. Several differences between the groups with and without MDS are apparent. Pregnant women with MDS also more often reported using medicines from integrative and complementary medicine than women without (**Table 4**).

Effectiveness and Tolerability of Candidate Herbal Medicines for MDS Treatment

Some questions on the questionnaire specifically targeted the use of well-known plants for the treatment of mild MDS, namely St. John's wort, hops, valerian, lavender, and bryophyllum.

St. John's wort was taken by 3.5% of women who answered the corresponding questions (12/341), in half of the cases during

TABLE 2 | Use of pharmaceutical herbal medicines during pregnancy by participants with and without mental disorders and/or symptoms (MDS).

Medicine	Total users (*n* = 272)	With MDS (*n* = 162)	Without MDS (*n* = 110)	*p*-value
	n/N*(%)[a]	n/N*(%)[a]	n/N*(%)[a]	
Ginger	121/246 (49.2)	71/147 (48.3)	50/99 (50.5)	0.734
Raspberry leaf	106/248 (42.7)	58/148 (39.2)	48/100 (48.0)	0.169
Bryophyllum	93/246 (37.8)	75/148 (50.7)	18/98 (18.4)	<0.001
Chamomile	66/243 (27.2)	40/144 (27.8)	26/99 (26.3)	0.794
Lavender	52/236 (22.0)	44/141 (31.2)	8/95 (8.4)	<0.001
Iron-rich herbs	30/243 (12.3)	19/144 (13.2)	11/99 (11.1)	0.628
Echinacea	19/232 (8.2)	13/138 (9.4)	6/94 (6.4)	0.408
Lemon balm	19/232 (8.2)	9/137 (6.6)	10/95 (10.5)	0.280
Valerian	11/234 (4.7)	10/140 (7.1)	1/94 (1.1)	0.031
St. John's wort	7/231 (3.0)	6/138 (4.3)	1/93 (1.1)	0.155
Horsetail	7/235 (3.0)	5/141 (3.5)	2/94 (2.1)	0.531
Passionflower	5/235 (2.1)	5/140 (3.6)	0/95 (0.0)	0.063
Hops	4/231 (1.7)	4/137 (2.9)	0/94 (0.0)	0.095
Horse chestnut	4/229 (1.7)	3/135 (2.2)	1/94 (1.1)	0.510
Ginseng	4/231 (1.7)	1/137 (0.7)	3/94 (3.2)	0.159
Golden root	3/233 (1.3)	3/139 (2.2)	0/94 (0.0)	0.152
Others	23/113 (20.4)	13/72 (18.1)	10/41 (24.4)	0.421

[a]*Percentage values without considering missing data.*

Data are sorted by frequency of total herbal medicine users. The questionnaire also contained items on California poppy, winter cherry and kava; no participant reported their use.

TABLE 3 | The most commonly used teas during pregnancy considering mental disorders and/or symptoms (MDS).

Tea	Total users (*n* = 329)	With MDS (*n* = 172)	Without MDS (*n* = 157)	*p*-value
	n/N*(%)[a]	n/N*(%)[a]	n/N*(%)[a]	
Fennel	157/329 (47.7)	87/172 (50.6)	70/157 (44.6)	0.301
Chamomile	153/329 (46.5)	82/172 (47.7)	71/157 (45.2)	0.621
Raspberry leaf	150/329 (45.6)	76/172 (44.2)	74/157 (47.1)	0.552
Herbal mixture	130/329 (39.5)	69/172 (40.1)	61/157 (38.9)	0.782
Peppermint	122/329 (37.1)	70/172 (40.7)	52/157 (33.1)	0.175
Fruit mixture	104/329 (31.6)	56/172 (32.6)	48/157 (30.6)	0.672
Lime blossom	68/329 (20.7)	42/172 (24.4)	26/157 (16.6)	0.096
Verveine/verbena	62/329 (18.8)	31/172 (18.0)	31/157 (19.7)	0.609
Rosehip	58/329 (17.6)	36/172 (20.9)	22/157 (14.0)	0.095
Stinging nettle	55/329 (16.7)	25/172 (14.5)	30/157 (19.1)	0.216
Lady's mantle	38/329 (11.6)	24/172 (14.0)	14/157 (8.9)	0.148
Lemon balm	37/329 (11.2)	17/172 (9.9)	20/157 (12.7)	0.328
Orange blossom	36/329 (10.9)	20/172 (11.6)	16/157 (10.2)	0.663
Aniseed	28/329 (8.5)	17/172 (9.9)	11/157 (7.0)	0.439
Sage	26/329 (7.9)	17/172 (9.9)	9/157 (5.7)	0.159
Elderflower	23/329 (7.0)	12/172 (7.0)	11/157 (7.0)	0.997
Cumin	23/329 (7.0)	15/172 (8.7)	8/157 (5.1)	0.193
St. John's wort	14/329 (4.3)	7/172 (4.1)	7/157 (4.5)	0.870
Ginger	11/329 (3.3)	4/172 (2.3)	7/157 (4.5)	0.287
Horsetail	8/329 (2.4)	6/172 (3.5)	2/157 (1.3)	0.190
Valerian	3/329 (0.9)	2/172 (1.2)	1/157 (0.6)	0.613
Others	30/329 (9.1)	18/172 (10.5)	12/157 (7.6)	0.366

[a]*Percentage values without considering missing data.*

Data are sorted by frequency of use of teas by total population.

pregnancy weeks 28–42. Three women reported good to very good tolerability (**Figure 3**) and moderate to good effectiveness. St. John's wort was mostly taken for the treatment of mood disorders (4/12, 33.3%).

Hops was used by 2.3% of women (8/343), in all phases of pregnancy. Hops was tolerated well: three of eight women reported very good tolerability and one of eight reported poor tolerability. Seven of eight women evaluated its effectiveness as very good. The two most frequently reported indications were stress and restlessness (both 3/8, 37.5%), followed by sleep disorders (2/8, 25.0%).

Valerian was used most frequently in the last trimester (7/20, 35.0%). Overall, 20 women reported using valerian (20/344,

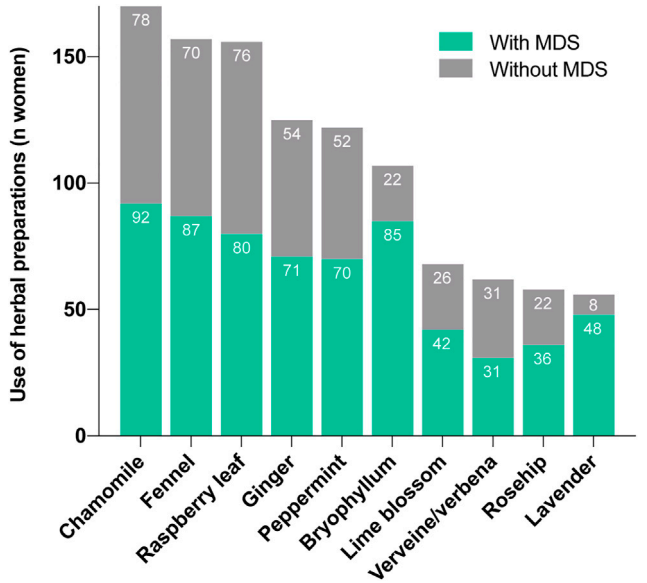

FIGURE 2 | Comparison of the most frequently used herbal medicines (pharmaceutical products and teas) in women with and without mental disorders and/or symptoms (MDS).

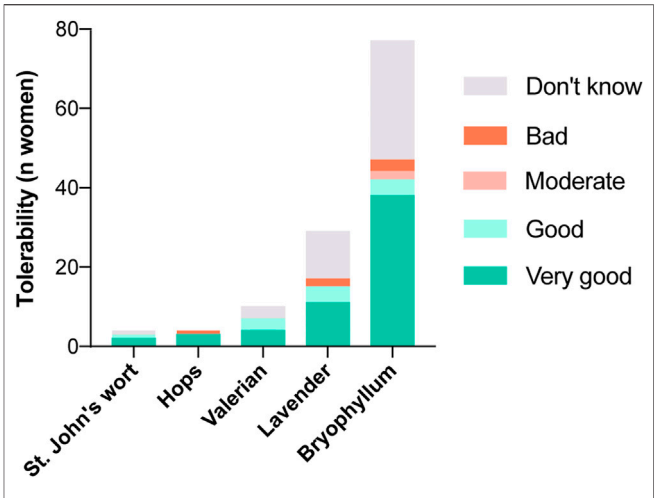

FIGURE 3 | Experiences with candidate herbal medicines for the treatment of mental disorders and/or symptoms (MDS) during pregnancy regarding their tolerability.

5.8%), none of the participants reported poor tolerability and the majority tolerated the herb well ("very good" 4/20, 20.0%; "good" 3/20, 15.0%). As shown in **Figure 4**, half of the participants rated its effectiveness as very good (10/20, 50.0%). Valerian was used most commonly to overcome sleep disorders (9/20, 45.0%), and restlessness (8/20, 40.0%).

The use of lavender was also increased toward the end of pregnancy. Lavender was used by 16.2% of women (56/345) and 11 of 56 women rated its tolerability as very good. Its effectiveness was described as good to very good by almost half of the women who used it (26/56, 46.4%), but bad by 35.7% of women (20/56). Lavender was used mostly to combat restlessness (28/56, 50.0%), sleep disorders (19/56, 33.9%) and stress (15/56, 26.8%).

Bryophyllum was used during pregnancy by almost one-third of the participants (107/360, 29.7%). Of these, more than three-quarters used it during pregnancy weeks 28–42 (84/107, 78.5%). Only a few women (3/107) reported poor tolerability. Regarding the effectiveness of bryophyllum, 58.8% of women rated it good to very good (63/107, all indications together; comparable percentages in the main single indications; see **Figure 4**). Restlessness was mentioned as a main indication by 27.1% of women (29/107) and 22.4% (24/107) used bryophyllum for stress relief. Another 10.3% (11/107) reported using bryophyllum for sleep disorders. Sixty-six percent of the women who had used

TABLE 4 | Use of medicines from integrative and complementary medicine during pregnancy.

	Total users (*n* = 116)	With MDS (*n* = 78)	Without MDS (*n* = 38)	*p*-value
	n/N*(%)[a]	n/N*(%)[a]	n/N*(%)[a]	
Anthroposophic medicine	73/326 (22.4)	51/78 (65.4)	22/38 (57.9)	0.759
Herbal medicines	72/336 (21.4)	51/78 (65.4)	21/38 (55.3)	0.600
Others	17/303 (5.6)	11/78 (14.1)	6/38 (15.8)	0.778
Not sure about intake	30/364 (8.2)	4/78 (5.1)	5/38 (13.2)	0.131
Homeopathy	61/330 (18.5)	40/78 (51.3)	21/38 (55.3)	0.418
Mother tinctures	19/352 (5.4)	14/78 (17.9)	5/38 (13.2)	0.579
Diluted herbal components	33/351 (9.4)	24/78 (30.8)	9/38 (23.7)	0.572
Others	28/320 (8.8)	16/78 (20.5)	12/38 (31.6)	0.259
Not sure about intake	18/370 (4.9)	13/78 (7.7)	2/38 (5.3)	0.684
Traditional Chinese medicine	24/336 (7.1)	17/78 (21.8)	7/38 (18.4)	0.735
Herbal medicines	21/353 (5.9)	16/78 (20.5)	5/38 (13.2)	0.331
Others	5/329 (1.5)	2/78 (2.6)	3/38 (7.9)	0.193
Not sure about intake	14/367 (3.8)	5/78 (6.4)	1/38 (2.6)	0.380
Ayurvedic medicine	12/337 (3.6)	8/78 (10.3)	4/38 (10.5)	0.910
Herbal medicines	12/351 (3.4)	8/78 (10.3)	4/38 (10.5)	0.959
Others	1/328 (0.3)	1/78 (1.3)	0/38 (0.0)	0.493
Not sure about intake	17/367 (4.6)	6/78 (7.7)	1/38 (2.6)	0.313

[a]*Percentage values without considering missing data.*

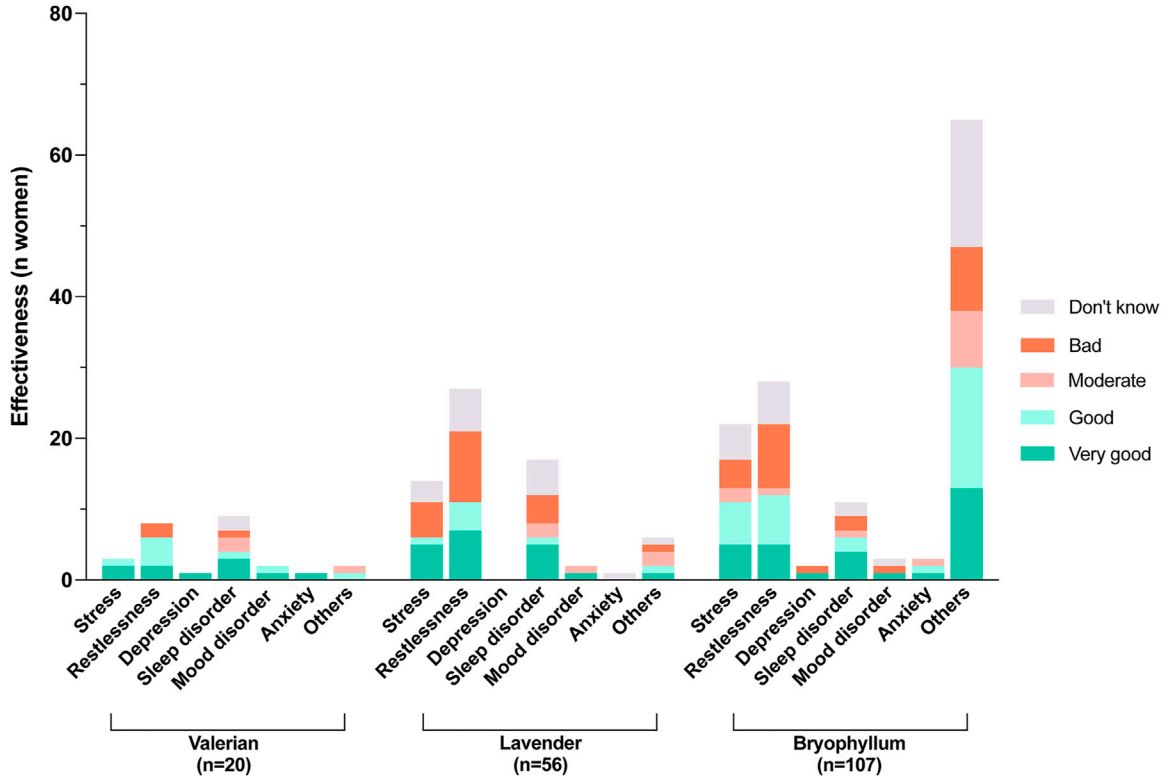

FIGURE 4 | Experiences with candidate herbal medicines for the treatment of mental disorders and/or symptoms (MDS) during pregnancy regarding their effectiveness.

bryophyllum (71/107, 66.4%) reported taking the herbal medicine for other reasons than those listed, and the majority wrote that the indications were any type of contractions (44/107, 41.1%). In addition, some women reported using bryophyllum for uterus soothing (n = 3), against abdominal cramps/pain (n = 3), against general pain (n = 1) and against cervix shortening (n = 1).

DISCUSSION

Main Findings

In our study population, the majority of women made use of herbal medicines, including pharmaceutical herbal products and teas. A wide variety of herbal products were mentioned by the survey participants, but ginger, raspberry leaf, bryophyllum, chamomile, lavender and iron-rich herbs were most commonly used. Our data further show that more than half (52.0%) of all participants had MDS during pregnancy, although few made use of (synthetic) psychoactive medications. The prevalence of MDS was higher among users of pharmaceutical herbal medicines than among non-users. Focusing on the most commonly used herbal medicines, users of bryophyllum and lavender reported suffering from MDS particularly often. Specific questions about candidate herbal medicines for the treatment of MDS revealed that bryophyllum (mentioned by 107 women), lavender (56 women) and valerian (20 women) were used to reduce stress, restlessness, sleep disorders and others, frequently with perceived good to very good effectiveness and tolerability.

Strengths and Limitations

All data were self-reported. While the number of pregnant participants (n = 398) can be considered a strength of the present survey, the moderate response rate of 24.1% is a limitation of the study. To avoid selections biases, following measures were taken: 1) all hospitals from the Canton of Zurich with an obstetrics ward were invited to participate; 2) the institutions that agreed to participate were regularly reminded of the survey; 3) the questionnaires were available in four languages (the three main country languages and English); 4) mental conditions were not mentioned in the title or cover letter of the questionnaire; 5) it was clearly stated that the survey was fully anonymous. Nevertheless, some selection bias due to interest in herbal medicines (mentioned in the title and cover letter) or the limited number of available questionnaire languages cannot be excluded. In the case of patients from the University Hospital Zurich (41.2% of survey participants), an internal analysis of the main patient demographic data revealed comparable characteristics to those of the survey participants (own unpublished observations). Finally, most questionnaires were handed over to women after delivery. While this can be

considered a strength–as postpartum women are able to describe their use of herbal medicines throughout the whole pregnancy–it is conceivable that some participants may not have remembered the medicines taken at the beginning of pregnancy.

Use of Pharmaceutic Herbal Medicines During Pregnancy

The high use of herbal medicines observed in the present survey is consistent with findings of a previous pilot survey conducted in a comparable population in the late 1990s (Gut et al., 2004). Studies from other countries reported lower use, and a multinational study of the use of herbal medications in pregnancy in 23 countries, and involving 9,459 women, revealed a markedly lower rate [28.9% of the women (Kennedy et al., 2013)]. This could be related to different perceptions of "herbal medicines" among participants in the different studies. In general, women in our study seemed to be more likely to use herbal medicines if they were primiparous and had used herbal medicines in the past/before pregnancy (data not shown). This could also be seen in an Australian study (Low Dog, 2009), where the most commonly used herbals were ginger, cranberry, valerian, raspberry, chamomile and peppermint (Kennedy et al., 2013), which is similar to our results.

In the following, we will focus on the herbal medicines used by at least 20% of the participants, first with respect to frequency of use during pregnancy and effectiveness, then summarising what is known on their safety. Our finding that ginger was the most commonly used herbal medicine confirms the results of previous surveys, and corresponds well with the high prevalence of nausea during pregnancy found in our survey (67.9%). Ginger can be considered a possibly effective treatment for nausea and vomiting during pregnancy (Viljoen et al., 2014). Raspberry leaf was the second most commonly used herbal medicine among pregnant women in our study, in accordance with studies showing that it is often recommended by midwives (Allaire et al., 2000; Holst et al., 2009). It is used to strengthen or prepare the uterus, to soften the cervix, and to induce and shorten labor (Briggs et al., 2022). In a placebo-controlled randomized trial, raspberry leaf tablets did not shorten the first stage of labor, but resulted in a small shortening of the second stage and less forceps deliveries (Simpson et al., 2001). Bryophyllum was the third most commonly used herbal medicine. Because this plant is recommended in several perinatal centres in Switzerland for the treatment of anxiety states, restlessness, and sleep disturbances (Schenkel et al., 2018), the questionnaire contained additional questions about its use (see below). Nevertheless, most women used bryophyllum for the treatment of other disorders, often related to the attenuation of uterine contractions. In anthroposophic medicine, bryophyllum was introduced in the 1970s as a well-tolerated agent for the treatment of preterm labor (Fürer et al., 2016; Hamburger et al., 2017); in Switzerland it is recommended for this indication (Schenkel et al., 2018) and commonly used in the main perinatal institutions (Fürer et al., 2015). Chamomile, the fourth most commonly used medicine has been also widely used and is known as a treatment for nausea and vomiting during pregnancy (Sanaati et al., 2016). Finally, lavender was frequently used (see below for information on its anxiolytic effects).

The Committee on Herbal Medicinal Products (CHMP) of the European Medicine Agency (EMA) does not recommend the use of ginger (European Medicines Agency, 2012b), raspberry leaf (European Medicines Agency, 2014), chamomile (European Medicines Agency, 2015) and lavender (European Medicines Agency, 2012a) during pregnancy because of insufficient safety data; so far, no community herbal monograph was published on bryophyllum. According to a reference work on drugs in pregnancy (Briggs et al., 2022), ginger and raspberry leaf are classified as compatible with pregnancy, whereas about chamomile it is considered that human data are limited and no relevant human data are available. According to a systematic review and meta-analysis from 2014, ginger can be considered a harmless treatment for nausea and vomiting during pregnancy: here no significant differences were found between the ginger and placebo treated groups for all reported adverse effects in various studies (Viljoen et al., 2014). A systematic review come to comparable conclusions (Stanisiere et al., 2018). With respect to raspberry leaf use, an unclear association with caesarean sections (Nordeng et al., 2011) and an interaction with a conventional medicine (hypoglycaemia when used with insulin) have been reported (Cheang et al., 2016). A randomized trial, however, revealed no adverse effects for mother or child (Simpson et al., 2001). Several retrospective and prospective studies on the use of bryophyllum during pregnancy are indicative of a good safety profile (Lambrigger-Steiner et al., 2014; Fürer et al., 2015; Fürer et al., 2016; Hamburger et al., 2017; Simões-Wüst et al., 2018). A qualitative study pointed towards an association between regular use of chamomile during pregnancy and a higher incidence of threatening miscarriages and preterm labor [without correction for possible confounders; Cuzzolin et al. (2010)].

Herbal Medicines and MDS Treatment

Of the herbal medicines specifically addressed in the present questionnaire, there were several that–irrespective of pregnancy–are used in the treatment of mild MDS. Evidence for their use during pregnancy is still scarce, therefore the existing studies are briefly discussed below (on the herbal medicines used by at least 20 participants).

Valerian was mostly used to treat restlessness and sleep disorders. As a sleep-aid, its benefits and side effect profile have been shown in several studies (Dorn, 2000; Andreatini et al., 2002; Hattesohl et al., 2008). In a small survey conducted in southern Italy, no influence of valerian use on pregnancy and neonatal outcomes was found ($n = 9$) (Low Dog, 2009). Data from the Swedish Birth Register from 1995 to 2004 also suggest good safety, as no abnormalities were found in the infants of mothers who had taken phytotherapeutics–often valerian–during pregnancy ($n = 787$, 0.9% of all mothers in the register).

In our study, women reported using **lavender** more often during the last trimester of pregnancy, mainly against

restlessness, sleep disorders and stress. Several clinical trials revealed good efficacy and tolerability of a medicine prepared from lavender flowers in the treatment of anxiety (Kasper et al., 2010; Woelk and Schlafke, 2010; Kasper et al., 2014). Furthermore, lavender tea has been shown to enhance the effects of the antidepressant citalopram (Nikfarjam et al., 2013). We are aware of only one previous study during pregnancy: in a randomised placebo-controlled trial, lavender cream was shown to reduce anxiety, stress and depression (Effati-Daryani et al., 2015).

Although most participants used **bryophyllum** to treat indications other than MDS, this plant was also used to treat restlessness, stress, sleep disorders, mood disorders, anxiety, and depression. In anthroposophic medicine, the use of bryophyllum medicines for mental disorders is well documented (Simões-Wüst et al., 2012). Prospective observational studies revealed improvements in sleep quality after treatment with bryophyllum in pregnant women (Lambrigger-Steiner et al., 2014) [and in cancer patients (Simões-Wüst et al., 2015)].

The particularly high prevalence of MDS among herbal medicine-users and the rare use of synthetic psychoactive medications suggest that pregnant women avoid them and prefer recurring to herbal medicines for mild MDS treatment. The reported positive experiences with some candidate herbal medicines for mild MDS treatment and their well-perceived tolerability deserve particular attention.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

GG and APS-W designed the study. GG developed the questionnaire, contacted the obstetric clinics and birth centres in the Canton of Zurich, performed the analyses of data on herbal medicines and wrote the first version of the manuscript. ER analysed the data on conventional medications and contributed to survey realization. DS and APS-W verified the analyses. All authors were involved in the interpretation of data, provided critical revision of the manuscript, then read and approved the submitted version. GG contribution constitutes her MD thesis, ER contribution constituted her Master's thesis in pharmacy; APS-W supervised both theses.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.729724/full#supplementary-material

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Conflict of Interest: APS-W received research funding from Weleda AG, a company that produces Bryophyllum pinnatum-based medicines during the last 5 years.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 1 – Supporting Information

Use of herbal medicines for the treatment of mild mental disorders and/or symptoms during pregnancy: A cross-sectional survey

Giulia Gantner, **Deborah Spiess**, Eliane Randecker, Katharina C. Quack Lötscher, Ana Paula Simões-Wüst

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Table 1S. General and pregnancy-related characteristics.

	All participants[a]	Herbal medicine users		
		Total	With MDS	Without MDS
n/%	398/100	358/100	192/100	166/100
Age (years)				
18-22	3/0.8	3/0.8	2/1.0	1/0.6
23-27	27/6.8	23/6.4	11/5.7	12/7.3
28-32	129/32.6	114/31.9	59/30.7	55/33.3
33-37	156/39.4	139/38.9	76/39.6	63/38.2
38-42	79/19.9	76/21.3	42/21.9	34/20.6
>43	2/0.5	2/0.6	2/1.0	0/0.0
Missing	2/-	1/-	0/-	1/-
Health insurance				
Basic	117/29.5	102/28.6	59/30.7	43/26.1
Basic+supplementary	195/49.2	176/49.3	95/49.5	81/49.1
Semi-private	61/15.4	58/16.2	28/14.6	30/18.2
Private	23/5.8	21/5.9	10/5.2	11/6.7
Missing	2/(0.5)	1/-	0/-	1/-
Marital status				
Married	273/68.9	242/67.8	120/62.5	122/73.9
Cohabitant	81/20.5	75/21.0	41/21.4	34/20.6
Single	41/10.4	39/10.9	30/15.6	9/5.5
Divorced/separated	1/0.3	1/0.3	1/0.5	0/0.0
Missing	2/-	1/-	0/-	1/-
Place of birth				
Switzerland	202/50.8	184/51.5	97/49.7	87/52.7
Germany	64/16.1	60/16.8	31/21.2	23/13.9
Other countries in Europe	86/21.6	71/19.8	37/19.3	34/20.6
America	19/4.8	18/5.0	7/3.7	11/6.6
Africa	2/0.5	2/0.6	1/0.5	1/0.6
Asia	16/4.0	15/4.2	8/4.2	7/4.2
Australia	1/0.3	1/0.3	1/0.5	0/0.0
Others	6/1.5	6/1.7	4/2.1	2/1.2
Missing	2/-	1/-	0/-	1/-
School education				
No school qualification	1/0.3	1/0.3	0/0.0	1/0.6
Secondary school	9/2.3	6/1.7	3/1.6	3/1.8
Professional apprenticeship	68/17.2	54/15.1	32/16.7	22/13.3
High school	23/5.8	20/5.6	12/6.3	8/4.8
Higher professional school	101/25.4	96/26.9	51/26.6	45/27.3
University	189/47.5	175/49.0	90/46.9	85/51.5
Others	5/1.3	5/1.4	4/2.1	1/0.6
Missing	2/-	1/-	0/-	1/-
Work situation at the beginning of pregnancy				
Employed in the health care sector	93/23.8	85/24.1	44/23.0	41/25.3
Employed in another sector	242/61.9	217/61.5	116/60.7	101/62.3
Housewife	23/5.9	20/5.7	10/5.2	10/6.2
Student	7/1.8	7/2.0	4/2.1	3/1.9
Self-employed or none of the above	19/4.8	18/5.1	14/7.3	4/2.5
Job seeker	7/1.8	6/1.7	3/1.6	3/1.9
Missing	7/-	5/-	1/-	4/-
Monthly gross income of household (CHF)				

<4'000	56/14.5	47/13.5	32/17.0	15/9.3
4'000-8'000	150/38.8	134/38.4	67/35.6	67/41.6
8'000-12'000	94/24.3	88/25.2	44/23.4	44/27.3
>12'000	86/22.3	80/22.9	45/23.9	35/21.8
Missing	12/-	9/-	4/-	5/-
Hospital				
University Hospital Zurich	164/41.5	147/41.3	82/42.7	65/39.6
City Hospital Triemli	61/15.4	55/15.4	28/14.6	27/16.5
Hospital Limmattal	21/5.3	18/5.1	6/3.1	12/7.3
Hospital Zollikerberg	50/12.7	47/13.2	27/14.1	20/12.2
Hospital Bülach	40/10.1	32/9.0	13/6.8	19/11.6
Paracelsus-Hospital Richterswil	31/7.8	31/8.7	19/9.9	12/7.3
Delphys Birthing Center	8/2.0	8/2.2	6/3.1	2/1.2
Others	10/2.5	9/2.5	5/2.6	4/2.4
Multiple	10/2.5	9/2.5	6/3.1	3/1.8
Missing	3/-	2/-	0/-	2/-
Language of the survey				
German	362/91.0	328/91.6	180/93.8	148/89.2
English	28/7.0	22/6.1	7/3.6	15/9.0
French	3/0.8	3/0.8	1/0.5	2/1.2
Italian	5/1.3	5/1.4	4/2.1	1/0.6
Pregnancy week				
28-37	79/21.3	75/22.4	51/28.3	24/15.5
38-42	71/19.1	65/19.4	36/20.0	29/18.7
Postpartum period	221/59.6	195/58.2	93/51.7	102/65.8
Missing	27/(6.8)	23/(6.4)	12/(6.3)	11/(6.6)
First pregnancy				
Yes	193/51.9	177/52.7	100/55.6	77/49.4
No	179/48.1	159/47.3	80/44.4	79/50.6
Missing	26/-	22/-	12/-	10/-
Number of previous pregnancies				
0	191/52.3	175/53.0	98/55.4	77/50.3
1	66/18.1	62/18.8	32/18.1	30/19.6
2	65/17.8	53/16.1	26/14.7	27/17.6
3	30/8.2	27/8.2	11/6.2	16/10.5
4	7/1.9	7/2.1	5/2.8	2/1.3
5	4/1.1	4/1.2	3/1.7	1/0.7
6	2/0.5	2/0.6	2/1.1	0/0.0
Missing	33/-	28/-	15/-	13/-
First child				
Yes	204/55.4	188/56.5	102/57.3	86/55.5
No	164/44.6	145/43.5	76/42.7	69/44.5
Missing	30/-	25/-	14/-	11/-
Recreational drug consumption in pregnancy				
Low alcohol consumption (<2 drinks/week)				
Yes	15/4.1	13/3.9	9/5.1	4/2.6
No	351/95.9	317/96.1	169/94.9	148/97.4
Missing	32/-	28/-	14/-	14/
Moderate alcohol consumption (2-4 drinks/week)				
Yes	0/0.0	0/0.0	0/0.0	0/0.0
No	366/100.0	330/100.0	178/100.0	152/100.0
Missing	32/-	28/-	14/-	14/-
Severe alcohol consumption (>5 drinks/week)				
Yes	1/0.3	1/0.3	1/0.6	0/0.0
No	365/99.7	329/99.7	177/99.4	152/100.0
Missing	32/-	28/-	14/-	14/-
Smoking				

Yes	15/4.1	15/4.5	7/3.9	8/5.3
No	351/95.9	315/95.5	171/96.1	144/94.7
Missing	32/-	28/-	14/-	14/-
Cannabis				
Yes	3/0.8	3/0.9	3/1.7	0/0.0
No	363/99.2	327/99.1	175/98.3	152/100.0
Missing	32/-	28/-	14/-	14/-
Cocaine				
No	366/100.0	330/100.0	178/100.0	152/100.0
Missing	32/-	28/-	14/-	14/-

[a] *All participants including users and non-users of herbal medicines.*

Table 2S. Acute and chronic disorders reported by pregnant women during pregnancy.

	Acute disorders		Chronic disorders	
	N	**% [a]**	**N**	**% [a]**
Any disorder	202	54.4	101	27.1
No disorder	169	45.6	272	72.9
Missing	27	-	25	-
Gastroesophageal reflux	69	18.6	n.a.	n.a.
Iron deficiency/anaemia	63	17.0	n.a.	n.a.
Sickness/vomiting/hyperemesis gravidarum	53	14.3	n.a.	n.a.
Diabetes	47	12.7	4	1.1
Obstipation	41	11.1	n.a.	n.a.
Piles/haemorrhoids	39	10.5	n.a.	n.a.
Headache/migraine	29	7.8	20	5.4
Sleeping disorder	27	7.3	1	0.3
Allergies	22	5.9	29	7.8
Thyroid disorder	21	5.7	24	6.4
Diarrhoea	21	5.7	n.a.	n.a.
Varicose veins	18	4.9	n.a.	n.a.
Gastrointestinal tract disorder	16	4.3	7	1.9
Mood disorder	13	3.5	5	1.3
High blood pressure	11	3.0	1	0.3
Low blood pressure	9	2.4	1	0.3
Urinary tract infection	9	2.4	n.a.	n.a.
Asthma	7	1.9	12	3.2
Minor depression	5	1.3	5	1.3
Anxiety disorder	3	0.8	3	0.8
Heart disorder	1	0.3	0	0.0
Rheumatic disorder	1	0.3	0	0.0
Kidney disorder	1	0.3	0	0.0
Epilepsy	0	0.0	2	0.5
Major depression	0	0.0	1	0.3
Cancer	0	0.0	1	0.3
Others	29	7.8	10	2.7

[a] *Percentage values without considering missing data.*

Multiple answers were possible. Data are sorted by frequency of acute disorders. n.a., not asked.

Table 3S. Reported symptoms during and before pregnancy.

	During pregnancy		Before pregnancy	
	N	% [a]	N	% [a]
Fatigue	294	79.5	84	22.7
Nausea	248	67.9	59	16.2
Heartburn	211	58.8	39	10.8
Shortness of breath	191	53.2	19	5.3
Constipation	166	48.3	89	25.9
Insomnia	145	42.9	50	14.8
Vomiting	128	38.3	63	19.0
Dizziness	96	29.3	55	16.7
Headache	95	28.4	122	36.5
Oedema	82	23.2	17	4.8
Abdominal pain	72	22.6	76	23.8
High heart rate	70	20.7	18	5.3
Anxiety	57	17.8	45	14.1
Depressive mood	33	9.9	43	12.8
Others	25	7.0	6	1.7

[a] *Percentage values without considering missing data.*

Multiple answers were possible. Data are sorted by frequency of symptoms during pregnancy.

Table 4S. Use of sedatives/anxiolytics and antidepressants during and before pregnancy.

	During pregnancy		Before pregnancy	
	n/N	% [a]	n/N	% [a]
Antidepressants	5/315	1.6	19/321	5.9
Sertralin	2/237	0.8	5/238	2.1
Citalopram	1/235	0.4	6/237	2.5
Others	2/240	0.8	9/242	3.7
Sedatives/anxiolytics	0/310	0.0	15/315	4.8
Lorazepam	0/242	0.0	11/243	4.5
Diazepam	0/243	0.0	3/243	1.2
Others	0/244	0.0	3/244	1.2

[a] *Percentage values without considering missing data.*

Chapter 2

Chapter 2

Medicinal plants for the treatment of mental diseases in pregnancy: An *in vitro* safety assessment

Deborah Spiess[*], Moritz Winker[*], Antoine Chauveau, Vanessa Fabienne Abegg, Olivier Potterat, Matthias Hamburger, Carsten Gründemann[**], Ana Paula Simões-Wüst[**]

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Pregnancy is a critical period for medical care, during which the well-being of woman and fetus must be considered. This is particularly relevant in managing non-psychotic mental disorders (NMDs) since treatment with central nervous system-active drugs and untreated NMDs may have negative effects. Some well-known herbal medicines (phytopharmaceuticals), including St. John's wort, California poppy, valerian, lavender, and hops possess antidepressant, sedative, anxiolytic, or antidepressant properties and could be used to treat mental diseases such as depression, restlessness, and anxiety in pregnancy. Our goal was to assess their safety in vitro, focusing on cytotoxicity, induction of apoptosis, genotoxicity, and effects on metabolic properties and differentiation in cells widely used as a placental cell model (BeWo b30 placenta choriocarcinoma cells). The lavender essential oil was inconspicuous in all experiments and showed no detrimental effects. At low-to-high concentrations, no extract markedly affected the chosen safety parameters. At an artificially high concentration of 100 μg/mL, extracts from St. John's wort, California poppy, valerian, and hops had minimal cytotoxic effects. None of the extracts resulted in genotoxic effects or altered glucose consumption or lactate production, nor did they induce or inhibit BeWo b30 cell differentiation. This study suggests that all tested preparations from St. John's wort, California poppy, valerian, lavender, and hops, in concentrations up to 30 μg/mL, do not possess any cytotoxic or genotoxic potential and do not compromise placental cell viability, metabolic activity, and differentiation. Empirical and clinical studies during pregnancy are needed to support these *in vitro* data.

Contributions of Deborah Spiess to this publication: designing and conducting in vitro assays focusing on metabolic activity and placental cell differentiation, analysis and interpretation of data, data visualization, writing the first manuscript draft, and manuscript revision

[*] These authors contributed equally to the work and should be considered as joint first authors

[**] These authors contributed equally to the work and should be considered as joint last authors

Medicinal Plants for the Treatment of Mental Diseases in Pregnancy: An *In Vitro* Safety Assessment

OPEN ACCESS

Authors

Deborah Spiess[1,2*], Moritz Winker[3,4*], Antoine Chauveau[2], Vanessa Fabienne Abegg[2], Olivier Potterat[2], Matthias Hamburger[2], Carsten Gründemann[3,4**], Ana Paula Simões-Wüst[1**]

Affiliations

1 Department of Obstetrics, University Hospital Zurich, University of Zurich, Zurich, Switzerland
2 Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland
3 Center for Complementary Medicine, Institute for Infection Prevention and Hospital Epidemiology, University of Freiburg, Faculty of Medicine, Freiburg, Germany
4 Translational Complementary Medicine, Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland

Key words

non-psychotic mental disorders, pregnancy, BeWo cell line, toxicity, *Hypericum perforatum*, Hypericaceae, *Eschscholzia californica*, Papaveraceae, *Valeriana officinalis*, Caprifoliaceae, *Lavandula angustifolia*, Lamiaceae, *Humulus lupulus*, Cannabaceae

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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 51 31, Fax: + 41 44 255 44 30
anapaula.simoes-wuest@usz.ch

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ABSTRACT

Pregnancy is a critical period for medical care, during which the well-being of woman and fetus must be considered. This is particularly relevant in managing non-psychotic mental disorders since treatment with central nervous system-active drugs and untreated NMDs may have negative effects. Some well-known herbal preparations (phytopharmaceuticals), including St. John's wort, California poppy, valerian, lavender, and hops, possess antidepressant, sedative, anxiolytic, or antidepressant properties and could be used to treat mental diseases such as depression, restlessness, and anxiety in pregnancy. Our goal was to assess their safety *in vitro*, focusing on cytotoxicity, induction of apoptosis, genotoxicity, and effects on metabolic properties and differentiation in cells widely used as a placental cell model (BeWo b30 placenta choriocarcinoma cells). The lavender essential oil was inconspicuous in all experiments and showed no detrimental effects. At low-to-high concentrations, no extract markedly affected the chosen safety parameters. At an artificially high concentration of 100 µg/mL, extracts from St. John's wort, California poppy, valerian, and hops had minimal cytotoxic effects. None of the extracts resulted in genotoxic effects or altered glucose consumption or lactate production, nor did they induce or inhibit BeWo b30 cell differentiation. This study suggests that all tested preparations from St. John's

* These authors contributed equally to the work and should be considered as joint first authors.

** These authors contributed equally to the work and should be considered as joint last authors.

wort, California poppy, valerian, lavender, and hops, in concentrations up to 30 µg/mL, do not possess any cytotoxic or genotoxic potential and do not compromise placental cell viability, metabolic activity, and differentiation. Empirical and clinical studies during pregnancy are needed to support these *in vitro* data.

ABBREVIATIONS

CHMP	Committee on Herbal Medicinal Products
CNS	central nervous system
CPT	camptothecin
EMA	European Medicines Agency
EMS	ethyl methanesulfonate
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
FSK	forskolin
LMA	low-melting agarose
NMA	normal-melting agarose
NMDs	non-psychotic mental disorders
SSRI	selective serotonin reuptake inhibitor
TX	Triton-X-100
***β*-hCG**	beta-human chorionic gonadotropin

Introduction

NMDs, such as depression and anxiety disorders, are common issues during pregnancy and the postpartum period. A recent prevalence estimate in Switzerland revealed that 17 % of women receive mental health care during pregnancy and the first postpartum year [1]. For women with pre-existing psychiatric conditions, this period is prone to recurrence or even worsening of NMDs. About 10–13 % of fetuses are exposed to a psychotropic drug [2], leading to side effects for both the mother and the fetus or newborn [3]. The most commonly used psychotropic medications include antidepressants, with the use of SSRIs such as citalopram being preferred [4]. Exposure to these medications and untreated depression in pregnancy have been associated with poor birth outcomes and increased risks, and the treatment of depression in pregnancy remains challenging [5, 6]. Benzodiazepines, particularly diazepam, are used to treat anxiety, sleep, and mood disorders. However, their use in the weeks before childbirth may provoke neonatal withdrawal syndrome, floppy infant syndrome, or various acute toxic effects in the newborn [7]. Since treating mental diseases during pregnancy with conventional medications has several drawbacks, and since an untreated NMD ifself should be avoided, safe herbal preparations may be a treatment option. Several herbal candidates could be considered to treat mild NMDs during pregnancy, namely hops, valerian, lavender, California poppy, and St. John's wort.

Preparations of hops (*Humulus lupulus* L., Cannabaceae) and valerian (*Valeriana officinalis* L., Caprifoliaceae) have a long history of traditional use for the treatment of sleeping disorders [8]. A noninterventional study of a combination of hops and valerian improved sleep latency and quality in adults with primary insomnia [8]. The combination also led to sleep-improving effects in a randomized, placebo-controlled sleep-EEG study [9]. However, divergent results have been reported regarding the clinical effectiveness of hop-valerian combinations (for a review, see [10]). In rodents, valerian showed anxiolytic and antidepressant-like activity, which may account for the sleep-enhancing effects of valerian [11]. Data from a Swedish birth register from 1995 to 2004 with 860′215 women show that valerian preparations are among the most frequently used medicines during pregnancy, and no unfavorable effects on pregnancy outcomes were reported [12]. A multinational study also showed that valerian is among the most frequently used herbal preparations in pregnancy [13]. In a recent retrospective observational study performed in the South of Italy, no influence of valerian ingestion on pregnancy and neonatal outcomes was detected (n = 9) [14]. However, to the best of our knowledge, no clinical or *in vivo* data are available on the safety of hops preparations in pregnancy.

The essential oil of lavender (*Lavandula angustifolia* Mill., Lamiaceae) is used in various applications and must contain 25–46 % linalyl acetate and 20–45 % linalool for therapeutic use [15]. It effectively treats anxiety [16], general restlessness, and difficulty falling asleep [15]. In adults with generalized anxiety disorder, the anxiolytic effect was comparable to that of a benzodiazepine (low-dose lorazepam [17] and the SSRI paroxetine [18]), and fewer adverse effects were reported than with the synthetic drugs.

California poppy (*Eschscholzia californica* Cham., Papaveraceae) is traditionally used to relieve mild symptoms of mental stress and as a sleeping aid [19]. Although herbal products have been on the market for almost 40 years (since 1982), there are very few clinical studies on their efficacy [20]. Anxiolytic and sedative effects have been reported in rodents [21], which could be explained by high affinity to benzodiazepine receptors of yet unknown compounds [22]. Various alkaloids have been identified in the aerial parts of California poppy but do not seem to bind to $GABA_A$ receptors and thus to modulate chloride currents [23].

Phytomedicines containing St. John's wort (*Hypericum perforatum* L., Hypericaceae) are widely used for the treatment of mild to moderate depression [24], nervous unrest, anxiety, and, to some extent, insomnia [15]. A meta-analysis of 27 studies revealed that the herbal antidepressant did not differ in efficacy from SSRIs [25]. Treatment of mild-to-moderate depression with St. John's wort extract resulted in fewer adverse events and thus fewer treatment discontinuations [25]. However, several relevant drug interactions with St. John's wort extract must be considered in clinical practice [26]. The potential risk for pregnant women is still unclear due to the current lack of clinical data and equivocal results in animal studies. Data on the prevalence of fetal malformations in pregnant women exposed to St. John's wort preparations are not extensive. A study using data from the Danish National Birth Cohort reported a nonsignificantly higher prevalence of malformations (8.1 %; 3/38) in the group exposed to St. John's wort than in the comparator group (3.3 %, 2′891/90′128, p = 0.13).

However, the difference was based on 3 cases only and did not follow a specific pattern [27]. Another prospective study compared the rate of major malformations in subjects taking St. John's wort (5.3%; 2/38) with pregnant women treated with another antidepressant (4.2%; 2/48) or healthy women (0%; 0/56). Similarly, the number of preterm and live births was comparable in all 3 groups [28].

A considerable proportion of healthcare professionals who deal with pregnant women daily recommend herbal medications [29]. Pregnant women themselves often turn to phytomedicines [30] and tend to perceive herbal products as safe [31]. However, the CHMP of the EMA does not recommend the use of hops [32], valerian [33], lavender [34], California poppy [19], and St. John's wort [35] during pregnancy because of a lack of sufficient safety data. Specifically, it is unknown to what extent phytochemicals cross the placenta barrier or interfere with placenta function and, thereby, may interfere with the development of the fetus.

Assessing the safety of phytopharmaceuticals is a significant challenge. The active ingredients of herbal extracts are multicompound mixtures, many of which may be metabolized by the intestinal microbiota upon oral administration or by the liver once they have been absorbed. An interdisciplinary project is underway to fill this gap and address some of these issues, ranging from metabolizing phytochemicals by intestinal microbiota to intestinal absorption, liver metabolism, and passage across the placental barrier [36]. We performed an *in vitro* assessment in BeWo cells of the safety profile of medicinal plants that are used to treat mild NMDs. This human choriocarcinoma cell line (clone b30) is a widely used *in vitro* model for investigating placental metabolism, villous trophoblast fusion, syncytium formation, and monolayer permeability. The undifferentiated, mononuclear cells (villous cytotrophoblasts) can undergo fusion and morphological differentiation into a layer of syncytiotrophoblasts with the addition of FSK [37]. The formation of a layer of syncytiotrophoblasts and the concomitant production of β-hCG are essential for the function of the human placenta and the preservation of a healthy pregnancy [37]. We assessed the cytotoxicity and genotoxicity of the extracts in BeWo b30 cells, their effects on critical metabolic properties such as glucose consumption and lactate production, and the ability to induce or inhibit cell differentiation.

Results

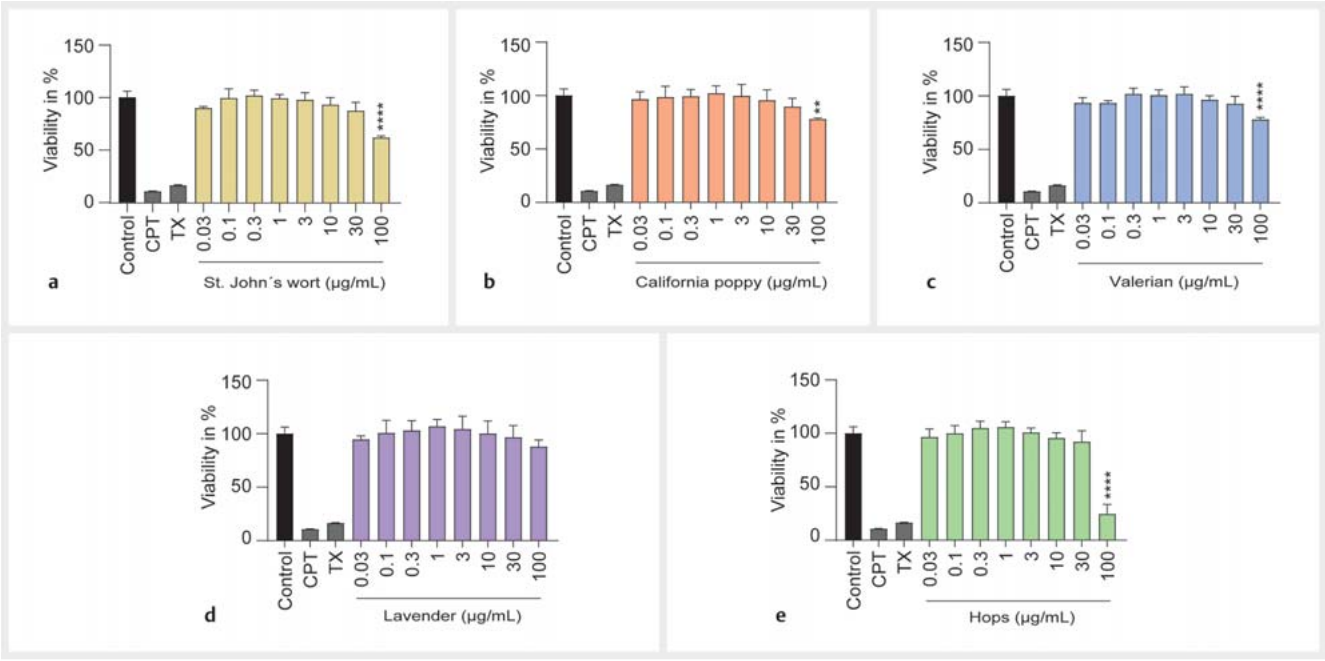
In herbal extracts, defining a concentration range for *in vitro* testing that reflects clinically attainable tissue concentrations is challenging. Maximum daily recommended doses for phytomedicines containing St. John's wort, hops, valerian, California poppy, and lavender essential oil range between 80 and 1200 mg/day (highest value corresponding to maximal dose of Somnofor; see [38]). Assuming a daily dose of 1200 mg extract dissolved in body-water (ca. 30.6 L in 15–24-year-old women, calculated for an average body weight of 60 kg [39]), an absence of metabolization by the intestinal microbiota, and a 100% bioavailability of all compounds, a maximal concentration of 39 µg/mL of extract would be reached in the body fluid. Based on this calculation, a concentration range of 0.03 to 100 µg/mL was used in the assays, whereby the higher test concentrations were significantly above possible clinically achieved tissue concentrations.

First, the effects of the extracts on cell viability and apoptosis induction were assessed via the turnover of WST-1 in viable cells. At concentrations of 30 µg/mL, all extracts showed no or only minimal cytotoxicity after 72 h of incubation, and pronounced cytotoxic effects for 4 out of 5 extracts were only observed at a concentration of 100 µg/mL (▶ **Fig. 1**). At this concentration, St. John's wort, California poppy, and valerian extracts reduced cell viability by 25–40% compared to untreated control cells, while hops extract lowered cell viability by 75%. Apoptosis in BeWo b30 cells was assessed via flow cytometric analysis. The extracts of California poppy and lavender did not induce apoptosis at concentrations up to 100 µg/mL (▶ **Fig. 2**). Extracts of St. John's wort, valerian, and hops only increased apoptotic cell death at the highest concentration of 100 µg/mL. Diazepam and citalopram were also tested for comparison and did not show cytotoxicity in this concentration range (**Fig. 8S**, Supporting Information).

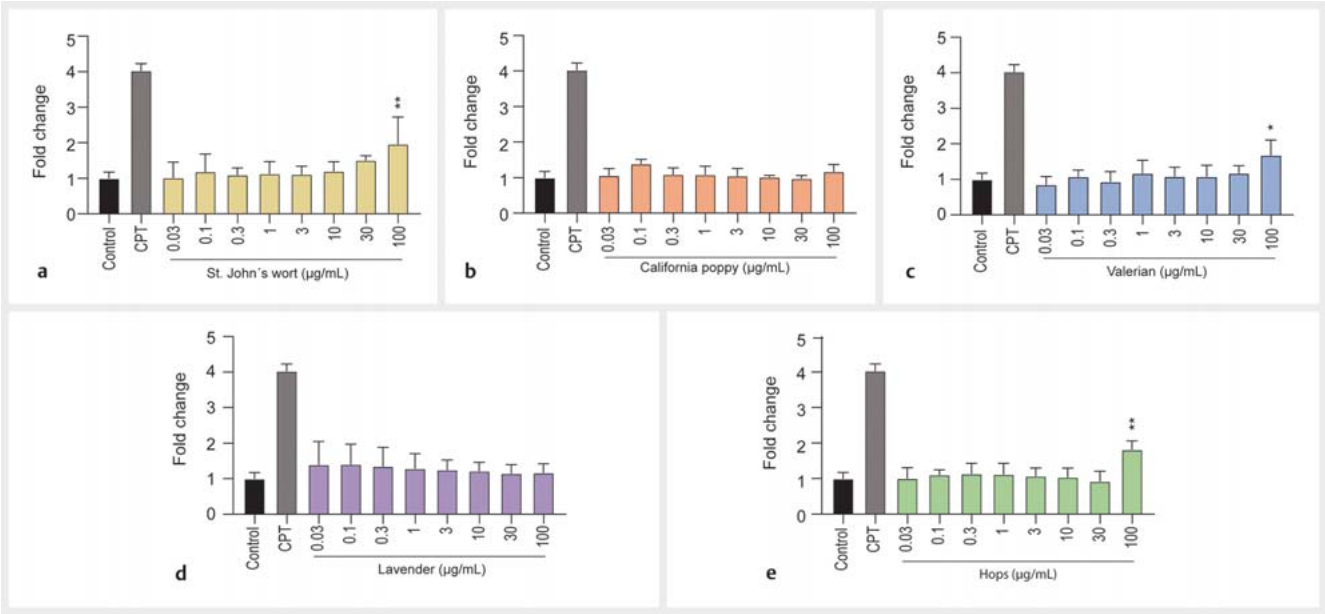
Possible genotoxicity of extracts was assessed with the aid of the comet assay, whereby a noncytotoxic concentration range (3, 10, and 30 µg/mL) was tested. None of the extracts induced notable increases in tail DNA, and mostly intact nuclear DNA could be detected after treatment of the cells with extracts for 3 h (▶ **Fig. 3**). Diazepam and citalopram also showed no genotoxicity in this assay (**Fig. 8S**, Supporting Information).

In the next step, the effect of the herbal extracts on glucose consumption and lactate production was examined. None of the extracts affected the metabolic activity of viable BeWo b30 cells when tested at concentrations up to 100 µg/mL. Glucose and lactate concentrations of cell supernatants were not statistically different from the untreated control (▶ **Fig. 4**). Data were normalized to the protein content. Without the normalization, valerian extract at a 100 µg/mL concentration led to a decrease in glucose consumption and concomitant reduction in lactate production (data not shown). Diazepam and citalopram did not lead to changes in glycolytic metabolism (**Fig. 8S**, Supporting Information).

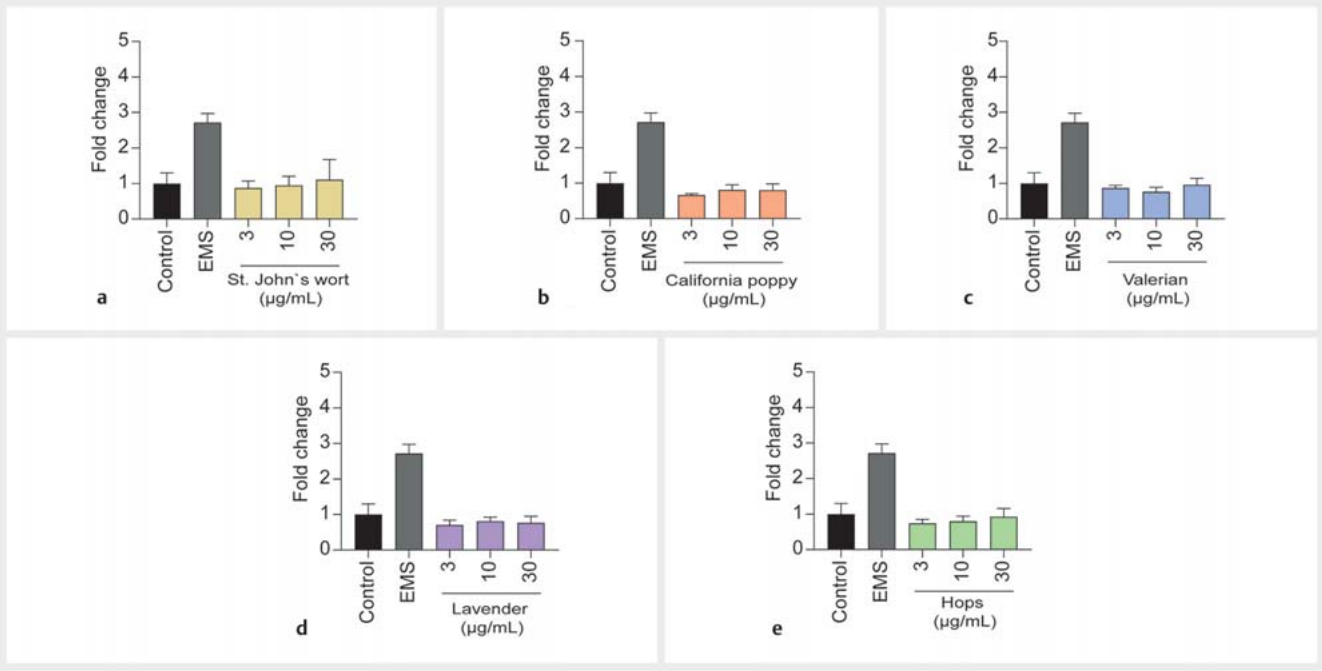
Finally, the impact of the extracts on β-hCG secretion was investigated. Upon adding 50 µM FSK as a positive control, a 100-fold increase of β-hCG levels was observed, which is characteristic of differentiation of BeWo b30 cells. In contrast, none of the 5 herbal extracts triggered an increase in β-hCG production (▶ **Fig. 5a**). Also, no significant decrease of β-hCG levels was observed for cells treated with the extracts compared to untreated control. Only the valerian extract's highest test concentration (100 µg/mL) led to a slight (nonsignificant) reduction. Only nontoxic extract concentrations were chosen to inhibit placental cell differentiation by herbal preparations (≤ 30 µg/mL). The addition of 5 µM FSK resulted in 5-fold increased β-hCG levels, showing that differentiation of BeWo b30 cells was successful (as shown in ▶ **Fig. 5b**) even after using 10-fold lower FSK concentrations and cell exposure for 24 h only. A 24 h pre-incubation with any of the 5 herbal preparations (in concentrations of 1, 3, 10, and 30 µg/mL) did not have a statistically significant inhibitory effect on the FSK-induced placenta cell differentiation (▶ **Fig. 5b**).



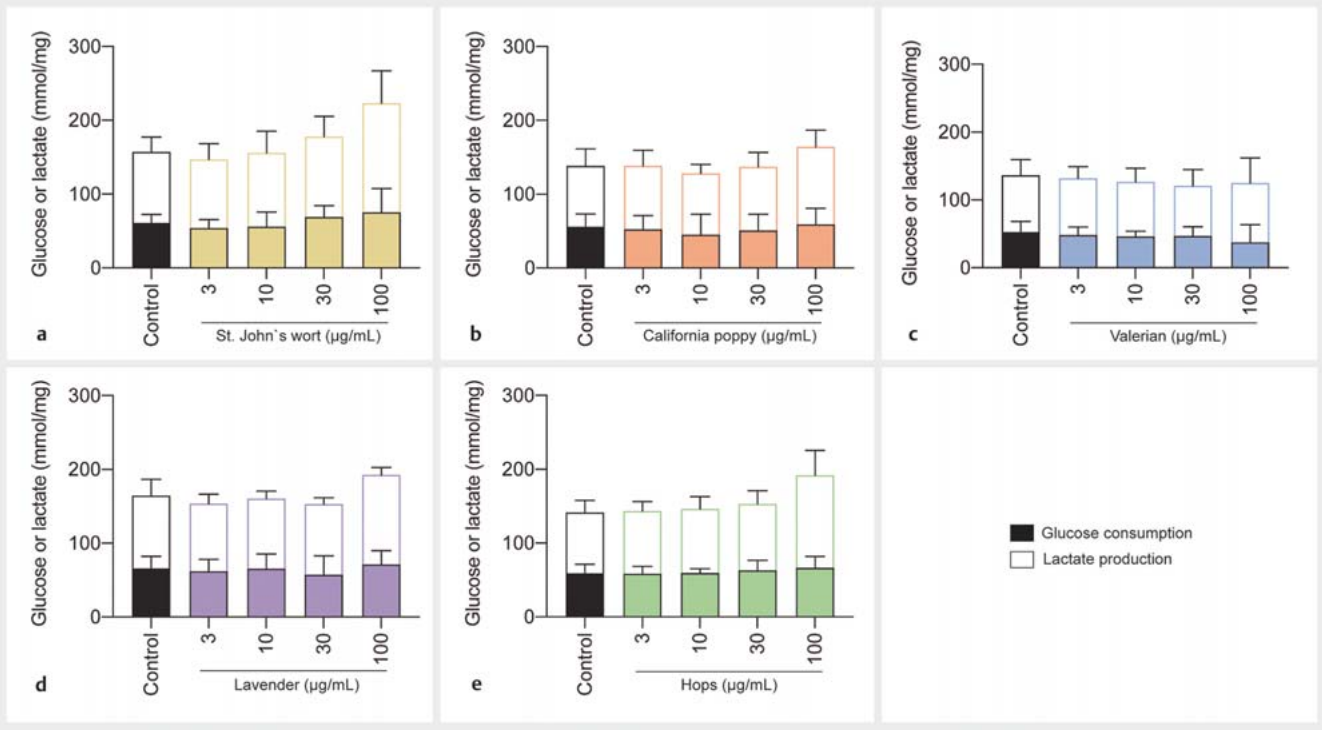
▶ **Fig. 1** Effects of extracts on cell viability of undifferentiated BeWo b30 cells. Cell viability was assessed with a WST-1 assay after 72 h of treatment. None of the extracts showed a significant effect at concentrations up to 30 µg/mL. Only the highest concentration of 100 µg/mL led to effects for 4 extracts St. John's wort (**a**), California poppy (**b**), valerian (**c**), and hops (**e**). Lavender oil did not lead to any significant effect (**d**). The effects are shown as fold change compared to the untreated control. Treatments with 300 µM CPT and 0.5% Triton-X-100 (TX) served as toxicity controls. Results were normalised to untreated control signal = 100% (n = 3).



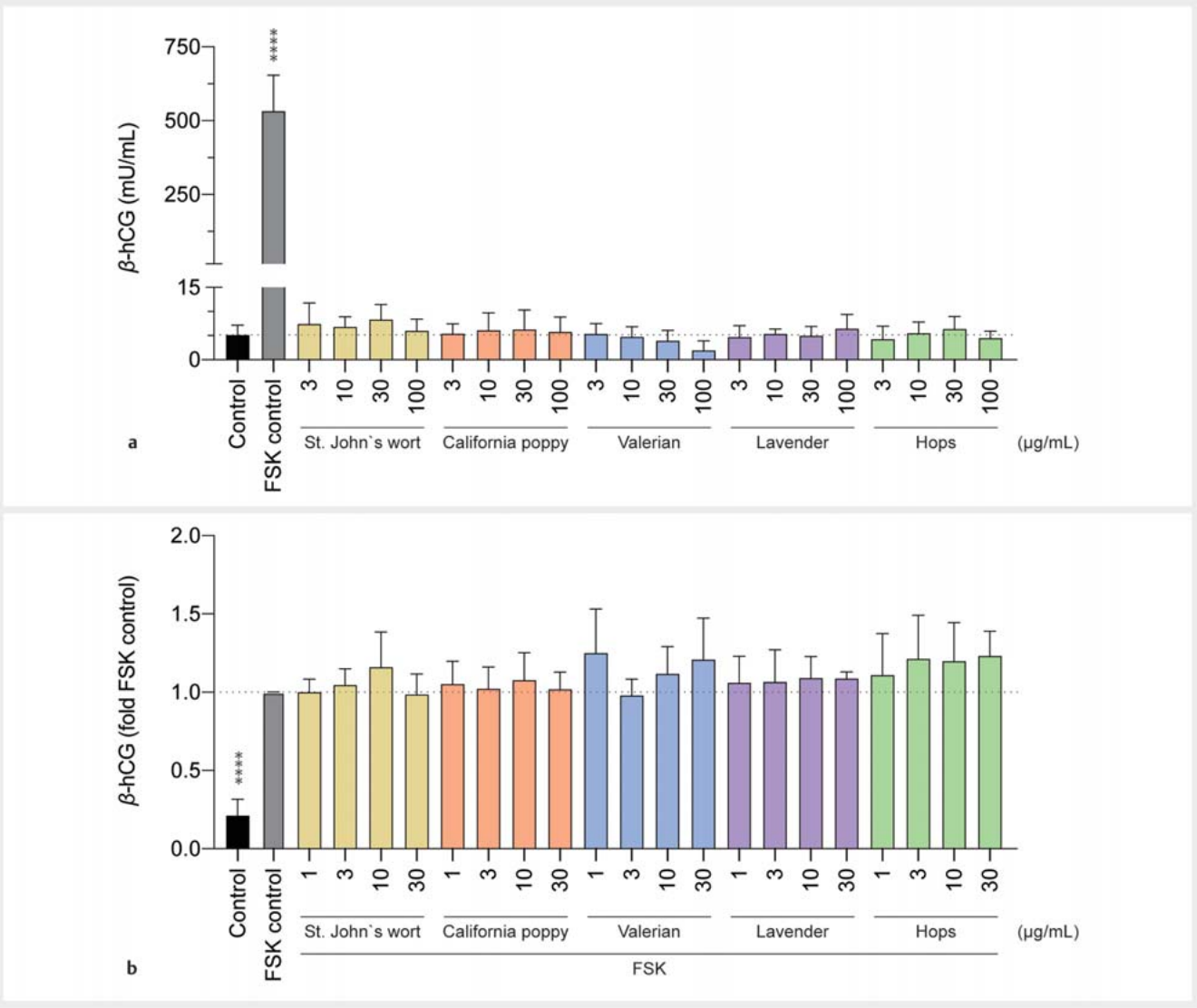
▶ **Fig. 2** Effects of extracts on cell death of undifferentiated BeWo b30 cells after treatment for 72 h. Apoptosis only significantly increased for the highest concentrations of St. John's wort (**a**), valerian (**c**), and hops (**e**). California poppy (**b**) and lavender (**d**) did not induce apoptosis at concentrations up to 100 µg/mL. Results were calculated as fold change compared to the untreated control. Camptothecin (CPT, 300 µM) was used as a positive control for apoptosis (n = 3).



▶ **Fig. 3** Effects of extracts of St. John's wort (**a**), California poppy (**b**), valerian (**c**), lavender (**d**), and hops (**e**) on tail DNA in undifferentiated BeWo b30 cells after exposure for 3 h. No significant genotoxic effects were observed at extract concentrations ranging from 3 to 30 µg/mL. Results were calculated as fold change compared to the untreated control. Ethyl methanesulfonate (EMS, 3 mM) was used as a positive control (n = 3).



▶ **Fig. 4** Effects of extracts on glucose consumption and lactate production in undifferentiated BeWo b30 cells after treatment for 48 h. Data were normalized per amount of protein (mg). Statistically significant impairment of metabolic activity could not be detected at all test concentrations (3, 10, 30, 100 µg/mL) of extracts of St. John's wort (**a**), California poppy (**b**), valerian (**c**), lavender (**d**), and hops (**e**). The control consisted of cell culture media containing 0.2% of DMSO. Data were obtained from 3 independent experiments (n = 3; in triplicate) and are shown as mean ± SD.



▶ **Fig. 5** Effects of extracts on the production of β-hCG in BeWo b30 cells. The control consisted of cell culture media containing 0.2 % of DMSO. Data are presented as mean ± SD of at least 3 independent experiments (n = 3–4; in triplicate). **a** Comparison of β-hCG secretion of BeWo b30 cells upon 48 h treatment with increasing concentrations of test compounds vs. 50 µM FSK control. **b** Effects on inhibition of FSK-induced differentiation of BeWo b30 cells (detected by measuring β-hCG). Treatment with 5 µM FSK led to increased β-hCG levels in all test compounds after exposure to different concentrations (1, 3, 10, 30 µg/mL) after an incubation of 48 h. Cells were pre-treated with the test compounds (or cell culture medium) for 24 h, before adding 5 µM FSK for another 24 h.

Discussion

Hydroalcoholic extracts from St. John's wort, California poppy, valerian and hops, and lavender essential oil did not induce *in vitro* cytotoxicity, apoptosis, or genotoxicity in BeWo b30 cells at concentrations up to 30 µg/mL. Moreover, there were no abnormalities in metabolic properties and no impact on cell differentiation. No changes were observed when normalizing the glucose and lactate concentrations (mmol) to the amount of protein (mg). This indicates that viable cells had a normal glycolytic metabolism under a 48 h exposure to extract concentrations up to 100 µg/mL. No significant β-hCG release was observed for all extracts, suggesting that they did not induce the cell fusion process. No significant decrease in β-hCG supernatant concentrations was seen when BeWo b30 cells were pre-incubated for 24 h with non-cytotoxic concentrations (≤ 30 µg/mL) of the extracts. These results showed that the extracts could not inhibit the cell differentiation of cytotrophoblasts into syncytiotrophoblasts. Reduced cell viability and induction of apoptosis were seen at the highest extract concentration of 100 µg/mL, except for lavender essential oil, which was inconspicuous in all experiments. To summarize, all herbal extracts of St. John's wort, California poppy, valerian, lavender, and hops showed no toxicological abnormalities in a relevant concentration range.

The main strength of this *in vitro* study is the combination of different and well-recognized assays to assess the safety of com-

monly used herbal preparations. A wide range of concentrations and various time exposures of up to 3 days were tested using the cell line most frequently used as a cellular model of placenta, which closely reflects the biological environment. The constraint to *in vitro* model is, at the same time, a limitation, which is quite difficult to overcome and translate into the clinical situation. For instance, intestinal and hepatic metabolism, clearance, allergic reactions, and pregnancy-related specificities could not be considered.

In our study, St. John's wort showed no significant effects at concentrations up to 30 µg/mL in all experiments. The viability assay and the apoptosis markers showed an impairment of the BeWo b30 cells only at the highest concentration of 100 µg/mL. This is in good agreement with other published *in vitro* data, where the toxicity of St. John's wort was seen at high concentrations (≥ 150 µg/mL) [40]. Moreover, no effect on the *β*-hCG production could be detected at 25 µg/mL of the extract [40]. Hyperforin reportedly inhibited the growth of embryonic stem cells and induced apoptosis in fibroblasts and thus may, at high concentrations, pose embryotoxic and teratogenic risks [41]. It should be added that the hyperforin content in phytomedicines varies widely, depending on the specific product [42].

We only found minimal cytotoxic potential for California poppy, as only the viability assay showed a slight significance at 100 µg/mL. Currently, there is a lack of sufficient clinical data on the safety of California poppy, as preparations have not been tested for reproductive toxicity, genotoxicity, and carcinogenicity. Furthermore, no data on *in vitro* cytotoxicity, genotoxicity, or influence on glycolytic metabolism and placental cell differentiation could be found. With our data, we have managed to provide an insight into *in vitro* safety that no one has found before to the best of our knowledge.

Valerian has been repeatedly discussed regarding a possible genotoxic potential. In a human endothelial cell line, 5–60 µg/mL of a dichloromethane extract of valerian show no significant cytotoxic effects. Discrete DNA damage occurred after *in vitro* exposure for 48 h to 40 or 60 µg/mL extract, but not at concentrations ≤ 40 µg/mL [43]. Valepotriates were considered responsible for the DNA damage [43]. However, valepotriates are unstable and degrade during the drying and heating of roots, and the valerian extract used in our study was devoid of valepotriates (see **Fig. 1S, Table 1S**, Supporting Information). *In vivo*, chromosomal aberrations, spermatozoa abnormalities, and a decrease in nucleic acids in testicular cells were reported to occur in mice after 7 days of oral administration of valerian by gavage (capsules containing 800 mg valerian root and 220 mg valerian root dried extract; dosage 500–2000 mg/kg/day) [44]. Valerian showed significant cytotoxic events in our setup for the same assays as St. John's wort but slightly less pronounced. It was also the only extract that had any significant effects on the metabolic properties of the BeWo b30 cells before normalizing per amount of protein available (data not shown). Valerian also showed a tendency (nonsignificant) to reduce *β*-hCG hormone concentrations in cell supernatants, which is most certainly due to cell toxicity and, therefore, decreased cell viability. Nevertheless, in all assays, the effects were only seen for the artificial nonphysiological concentration of 100 µg/mL. No significant effects could be detected at concentrations up to 30 µg/mL.

The essential oil of lavender did not show any impairment of the BeWo b30 cells at concentrations up to 100 µg/mL. How much of the volatile components of the oil were still present at the end of our experiments (48–72 h at 37 °C) is unknown and requires further clarification.

The hops extract affected cell viability and induced apoptosis only at a physiologically irrelevant concentration of 100 µg/mL. Only a few toxicity data on hops (compounds) are available. This is the case of prenylated flavonoids that revealed antiproliferative and cytotoxic effects in human cancer cell lines [45]. It must be noted that hop extracts are often combined with valerian or other extracts, and mono-preparations are not available in Switzerland.

Extracts of St. John's wort, California poppy, valerian, lavender, and hops do not appear to affect the functionality of placenta cells at concentrations that can be expected upon ingestion of recommended daily doses of phytomedicines. Only at very high test concentrations, particularly in the case of St. John's wort, a decrease of cell viability and induction of apoptosis was observed. Most importantly, no indications for genotoxic effects and no alterations in important metabolic parameters or cell differentiation were detected.

Materials and Methods

Plant material and extraction

All plant material was of Ph. Eur. grade. *V. officinalis* roots, *H. lupulus* flowers, and *H. perforatum* herb were purchased from Dixa (lot numbers 180 084, 191 241, 192 140, respectively). *E. californica* herb was obtained from Galke (lot number 811 502). Voucher specimens (numbers 1029, 1167, 1166, and 1234, respectively) have been deposited at the Division of Pharmaceutical Biology, University of Basel. The powdered plant material was extracted with 70 % EtOH by pressurized liquid extraction in a Dionex ASE 200 Accelerated Solvent Extractor. Three cycles of extraction of 5 min each were performed at a temperature of 70 °C and a pressure of 120 bar. *L. angustifolia* essential oil Ph. Eur. was purchased from Hänseler (lot number 2018.01.0274).

HPLC-PDA-ESI-MS analysis of herbal extracts

HPLC-PDA-ESI-MS analysis of *V. officinalis* (**Fig. 1S, Table 1S**, Supporting Information), *H. lupulus* (**Fig. 2S, Table 2S**, Supporting Information), *E. californica* (**Fig. 3S, Table 3S**, Supporting Information), and *H. perforatum* (**Fig. 4S, Table 4S**, Supporting Information) 70 % EtOH extracts were performed on an LC-MS system consisting of an 8030 triple quadrupole MS connected to an HPLC system consisting of a DGU-20A degasser, an LC-20AD binary high-pressure mixing pump, a SIL-20 ACHT autosampler, a CTO-20AC column oven, and an SPD-M20A diode array detector (all Shimadzu). The mobile phase consisted of water (A) and acetonitrile (B), both containing 0.1 % formic acid. Analyses were performed at 25 °C on a SunFire C18 column (3.5 µm; 150 × 3 mm i. d., Waters). *V. officinalis* and *H. lupulus* extracts were analyzed with a 5–100 % B gradient in 30 min at flow rates of 0.4 mL/min or 0.5 mL/min, respectively. *E. californica* extract was analyzed with a 5–40 % B gradient in 30 min at a 0.5 mL/min flow rate. *H. perforatum* extract was analyzed with a gradient of 10–23 % B

in 20 min, then 23–70 % B in 10 min at a flow rate of 0.4 mL/min. Extracts were dissolved in DMSO at a concentration of 10 mg/mL. The injection volume was 10 µL. Compounds were identified based on UV and MS spectroscopic data. The identity of compounds was further confirmed by chromatographic comparison with reference compounds when available.

HPLC-UV analysis of hyperforin, adhyperforin, hypericin, and pseudohypericin in *H. perforatum*

HPLC-UV analysis was performed on an Alliance 2690 chromatographic system coupled to a PDA996 detector (Waters). The mobile phase consisted of water (A) and acetonitrile (B), both containing 0.1 % trifluoroacetic acid (hyperforin and adhyperforin), or 0.5 % trifluoroacetic acid (hypericin and pseudohypericin). Separation of hyperforin and adhyperforin was achieved on a Zorbax Eclipse XDB-C8 column (3.5 µm; 150 × 2.1 mm i. d., Agilent) with a gradient of 50–100 % B in 20 min at a flow rate of 0.4 mL/min (**Fig. 5S**, Supporting Information). Hypericin and pseudohypericin were analyzed on an Atlantis dC18 column (3 µm; 150 × 4.6 mm i. d., Waters) with a 45–100 % B gradient in 15 min (**Fig. 6S**, Supporting Information). The extract was dissolved in DMSO at a concentration of 10 mg/mL. The injection volume was 10 µL. The identity of hyperforin, hypericin, and pseudohypericin was confirmed by chromatographic comparison with reference compounds.

GC-MS analysis of *L. angustifolia* essential oil

GC-MS analysis was performed on a Hewlett-Packard G1503A GC/MS system equipped with a 5973 Mass Selective Detector and a 59 864B Ion Gauge Controller (all Agilent). A J&W 122–5536 GC column (0.5 µm; 30 m × 0.25 mm i. d., Agilent) was used, with helium (1.4 mL/min) as a carrier gas. The injector temperature was set at 280 °C, and the transfer line temperature was 240 °C. The following temperature program was applied: 60 °C hold for 1 min, linear increase to 240 °C at 10 °C/min, followed by 5 min at 240 °C. EI ionization was in positive ion mode (electron energy: 2040 V; Full Scan: m/z: 50–700). Linalool and linalyl acetate were identified by comparing their MS spectra with the NIST database (**Fig 7S, Table 5S**, Supporting Information).

Cell culture

The human choriocarcinoma BeWo cell line (clone b30) was obtained from Dr. Tina Buerki-Thurnherr (Empa – Swiss Federal Laboratories for Materials Science and Technology, St. Gallen, Switzerland), with permission from Dr. Alan L. Schwartz (Washington University School of Medicine, MO, USA). Cells were maintained in modified F-12K Nut Mix (Gibco). The cell culture medium was supplemented with 10 % heat-inactivated FBS, penicillin (100 U/mL) and streptavidin (100 µg/mL), and 1 % L-glutamine (all from Gibco). Cells were cultured in monolayers at 37 °C in a humidified atmosphere of 5 % CO_2 balance air, and the medium was changed every 2–3 days.

Cell treatments

Plant extracts and lavender essential oil were dissolved in sterile DMSO (Sigma-Aldrich) to obtain a final concentration of 50 mg/mL for the stock solutions. In each experiment, treatments and controls consisted of cells exposed to a final concentration of 0.2 % (vol/vol) DMSO to not decrease the cell viability (**Fig. 9S**, Supporting Information). If required, BeWo b30 cells were stimulated with 5 or 50 µM FSK (Lucerna-Chem). FSK was added to the medium directly from a DMSO stock solution (30 mM). For all assays, cells were treated with increasing concentrations of extracts (up to 100 µg/mL) in cell culture medium. Samples were always protected from light while using St. John's wort extract.

Cytotoxicity assay

The *in vitro* cytotoxicity of the different concentrations of herbal extracts (0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 µg/mL) was tested using a WST-1 viability assay. BeWo b30 cells were seeded with 100 µL/well (2×10^5 cells/mL) in a 96-well flat-bottom plate the day before, followed by exposure to extract dilutions in a fresh culture medium. Cells treated with 300 µM CPT (apoptosis control; Tocris Bioscience) or 0.5 % TX (necrosis control; Sigma-Aldrich) were used as positive controls. After an incubation period of 72 h at 37 °C and 5 % CO_2, the culture supernatant was aspirated and replaced by a medium without phenol red, and 5 µL Cell Proliferation Reagent WST-1 (Roche) was added. After 75 min of incubation at 37 °C and 5 % CO_2, a spectrophotometric measurement was taken at 450 nm using a plate reader (Tecan Reader Infinite M 200).

Apoptosis assay

To assess the level of apoptosis after application of the test substances, BeWo b30 cells were subjected to the same treatment described for the WST-1 assay. Each extract was prediluted in a 96-well V-bottom plate and then added to the BeWo b30 cell culture to achieve final concentrations. After 72 h, the cells were washed with PBS and detached using Accutase (Sigma-Aldrich). According to the manufacturer's instructions, all liquids were pooled to collect living cells and fragments, centrifuged at 300 g for 5 min, and then stained with AnnexinV-FITC (eBioscience). A FACS readout was obtained using a fluorescence-activated cell analysis (BD FACScalibur, BD Biosciences).

Comet assay

The genotoxic potential of the selected extracts was examined by conducting a comet assay. A short incubation time of 3 h was chosen to avoid the onset of cellular DNA-repair mechanisms. Before the experiment, microscopic slides were coated with 1.0 % NMA, and 0.7 % LMA (both by SERVA Electrophoresis GmbH) was prepared and stored at − 20 °C until use. The cell suspension (100 µL/well) was seeded a day before the experiment with a 4×10^5 cells/mL cell concentration. On the experiment day, different concentrations of herbal preparations (3, 10, and 30 µg/mL) or 3 mM EMS (positive control; Sigma-Aldrich) were added for 3 h. Then, 200 µL of a completely dissolved and slightly boiling 0.7 % NMA solution was applied to the precoated slides, which were cooled on metal plates in the fridge for later use. After 3 h, the cells were washed once with PBS and dissolved using Accutase (Sigma-Aldrich). The cells were then resuspended in 30 µL complete medium. The previously prepared LMA was rapidly heated to 100 °C and then kept at 38 °C. The cells were then gently mixed with 90 µL 0.7 % LMA and added as a final layer to the slides. After

the slides were cooled on the metal plates for 15 min, the slides were placed in a lysis solution in the refrigerator for 1 h, after which the DNA was exposed. The slides were then placed in the electrophoresis chamber and submerged with electrophoresis buffer. After 20 min accommodation time, electrophoresis was performed for 20 min at 25 V/300 mA. Finally, the slides were washed with ddH_2O and PBS and fixed with 99% EtOH. For the microscopic measurement, the fixed samples were stained with ethidium bromide solution (5 µg/mL; Carl Roth GmbH). Pictures were taken for later analysis with CometScore software (version 2.0.038 for Windows; TriTek Corp., USA).

Glucose and lactate concentration measurements

Cells were seeded into transparent 24-well plates at a density of 2.5×10^4 cells/1000 µL/well. After overnight incubation, they were exposed to different concentrations (3, 10, 30, and 100 µg/mL) of plant extracts, lavender essential oil, and untreated control (0.2% DMSO) for 48 h. Cell culture supernatants and pellets were collected and immediately frozen at − 80 °C for subsequent analysis. All experiments were performed 3 times independently (in triplicate). The metabolic parameters–glucose and lactate–were determined using an automated blood gas analyzer (ABL800 Flex, Radiometer Medical ApS) based on amperometric measuring principles.

Placental cell differentiation and β-hCG production

Two different setups were used to test the influence of plant extracts and lavender essential oil on the induction or inhibition of placental cell differentiation. For the induction setup, cells were seeded into transparent 24-well plates (2.5×10^4 cells/1000 µL/well) before exposure to different concentrations (3, 10, 30, and 100 µg/mL) of test compounds and control (0.2% DMSO) for 48 h the next day. FSK (50 µM) was used as the positive control. For the inhibition setup, cells were seeded with 100 µL/well (1×10^5 cells/mL) into transparent 96-well flat-bottom plates on the day before the stimulation with test compounds and cell culture medium. After 24 h incubation, cells were differentiated with 5 µM FSK for another 24 h. All experiments were performed at least 3 times independently (in triplicate). Analysis of β-hCG concentrations–a marker of placenta cell differentiation–was performed using cell culture supernatants by standard ELISA (see [36] for additional references). Transparent 96-well flat-bottom microplates were used for all analyses. Samples treated with 50 or 5 µM FSK were diluted 1 : 50 or 1 : 10, respectively, before analysis. Rabbit polyclonal anti-hCG antibody (Agilent Dako) was used at a 1 : 1′000 dilution; mouse monoclonal anti-hCG (abcam) and goat anti-mouse-IgG-horseradish peroxidase conjugate (abcam) antibodies were both used at a 1 : 5′000 dilution. The peptide hormone hCG (Lucerna-Chem) was used as reference standard.

Protein concentration

BeWo b30 cells were lysed and extracted in the radioimmunoprecipitation assay buffer (Thermo Scientific) solution with 0.1% protease and phosphatase inhibitor single-use cocktail and 0.1% EDTA solution (Thermo Scientific). The cellular protein concentration was determined in transparent 96-well flat-bottom microplates by spectrophotometric quantification at 562 nm using the bicinchoninic acid protein assay kit (Pierce) with BSA (Thermo Scientific) as reference standard.

Statistical data analysis

Statistical data analyses were performed using GraphPad Prism (version 8.4.3 for Windows or macOS; GraphPad Software, La Jolla CA, USA). Shapiro-Wilk test was used to check for normal distribution. Multiple group comparisons were performed using the Brown-Forsythe and Welch ANOVA tests, followed by the Dunnett's T3 multiple comparisons posthoc test (with individual variances computed for each comparison). Probability values $^*p \le 0.05$ were considered statistically significant. The asterisks represent significant differences from the control group ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). All results are expressed as means ± SD of at least 3 independent experiments.

Supporting Information

HPLC-UV-MS chromatograms of EtOH extracts of *V. officinalis, H. lupulus, E. californica, H. perforatum*; HPLC-UV chromatograms of *H. perforatum* extract; GC-MS chromatogram of *L. angustifolia* essential oil; all data obtained with diazepam and citalopram; and *in vitro* cytotoxicity of different concentrations of DMSO on the BeWo b30 cells are available as Supporting Information.

Contributors' Statement

APSW, CG, MH and OP designed the study. DS and MW planned and conducted the experiments, performed the data analysis and prepared the original manuscript. DS focused on the metabolic properties and differentiation of placenta cells, and wrote the first version of the manuscript. MW conducted experiments on the viability, apoptosis and genotoxicity of placenta cells. AC performed the HPLC-UV-MS and GC-MS analyses. VA prepared all extracts. All authors agreed with the final version.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Chapter 2 – Supporting Information

Medicinal plants for the treatment of mental diseases in pregnancy: An *in vitro* safety assessment

Deborah Spiess[*], Moritz Winker[*], Antoine Chauveau, Vanessa Fabienne Abegg, Olivier Potterat, Matthias Hamburger, Carsten Gründemann[**], Ana Paula Simões-Wüst[**]

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[*] *These authors contributed equally to the work and should be considered as joint first authors*

[**] *These authors contributed equally to the work and should be considered as joint last authors*

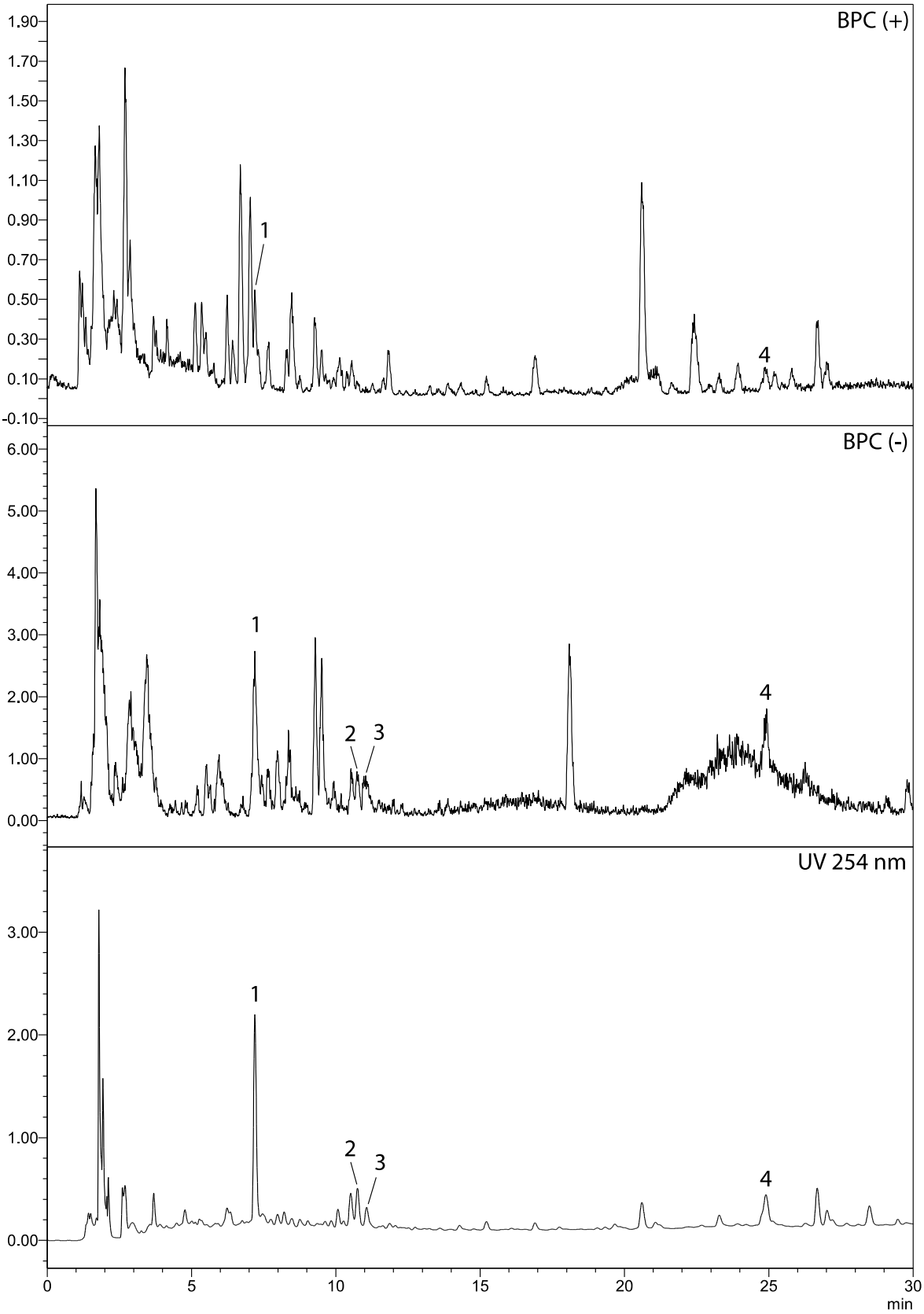

Figure 1S. HPLC-UV-MS analysis of *Valeriana officinalis* 70% EtOH extract. BPC: base peak chromatogram. **1:** chlorogenic acid, **2** and **3:** dicaffeoylquinic acid isomers (tentative assignment), **4:** valerenic acid.

Table 1S. Retention times and spectroscopic data of annotated peaks in *Valeriana officinalis* 70% EtOH extract.

Peak number	Retention time (min)	UV maxima (nm)	ESI MS (+) (m/z)	ESI MS (-) (m/z)	Identification
1	7.2	206, 310, 327	354.9 $[M+H]^+$	353.1 $[M-H]^-$	Chlorogenic acid
2	10.8	209, 310, 327	516.9 $[M+H]^+$ 499.0	515.1 $[M-H]^-$	Dicaffeoylquinic acid
3	11.1	217, 310, 327	516.9 $[M+H]^+$ 499.0	515.1 $[M-H]^-$	Dicaffeoylquinic acid
4	24.9	222	235.1 $[M+H]^+$	233.1 $[M-H]^-$	Valerenic acid

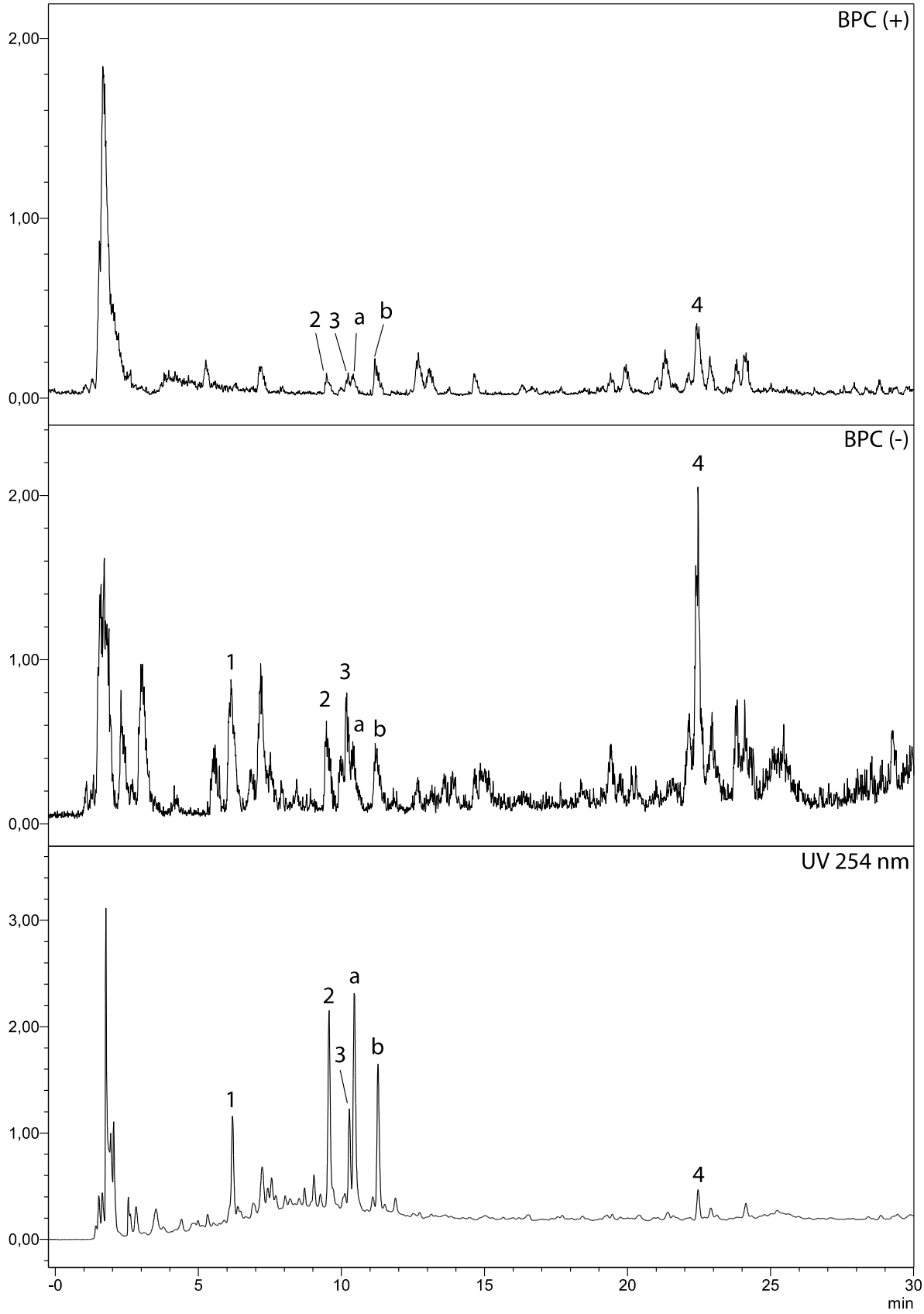

Figure 2S. HPLC-UV-MS analysis of *Humulus lupulus* 70% EtOH extract. BPC: base peak chromatogram. **1:** chlorogenic acid, **2:** isoquercitrin, **3:** kaempferol hexoside (tentative assignment), **4:** xanthohumol, **a:** unidentified flavonoid, MW 550 amu, **b:** unidentified flavonoid, MW 534 amu.

Table 2S. Retention time and spectroscopic data of annotated peaks in *Humulus lupulus* 70% EtOH extract.

Peak number	Retention time (min)	UV maxima (nm)	ESI MS (+) (m/z)	ESI MS (-) (m/z)	Identification
1	6.2	203, 300, 325	354.9 $[M+H]^+$	353.1 $[M-H]^-$	Chlorogenic acid
2	9.6	203, 255, 353	465.1 $[M+H]^+$	463.0 $[M-H]^-$	Isoquercitrin
3	10.3	201, 265, 343	448.9 $[M+H]^+$ 286.7	447.0 $[M-H]^-$	Kaempferol hexoside
4	22.5	222, 369	354.9 $[M+H]^+$	353.0 $[M-H]^-$	Xanthohumol
a	10.5	204, 255, 353	550.8 $[M+H]^+$	549.2 $[M-H]^-$	Unidentified flavonoid
b	11.3	205, 265, 346	534.9 $[M+H]^+$	533.0 $[M-H]^-$	Unidentified flavonoid

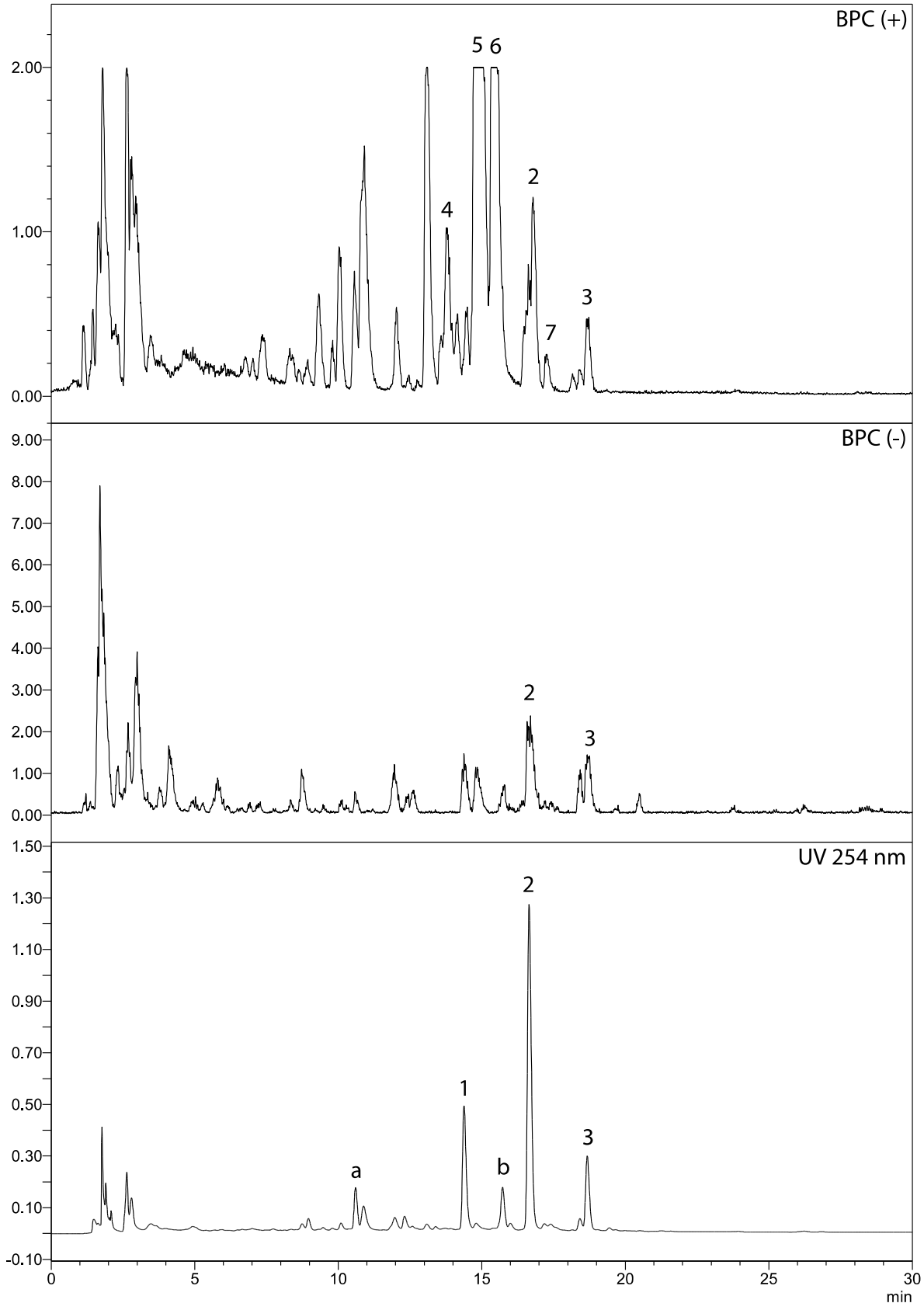

Figure 3S. HPLC-UV-MS analysis of *Eschscholzia californica* 70% EtOH extract. BPC: base peak chromatogram. **1:** rutin rhamnoside (tentative assignment), **2:** rutin, **3:** rhamnoside of *O*-methyl quercetin (tentative assignment), **4:** protopine, **5:** californidine, **6:** escholzine, **7:** neocaryachine-7-*O*-methyl ether *N*-metho salt (CAS-Nr. 1310405-84-5, tentative assignment), **a** and **b:** unidentified flavonoids.

Table 3S. Retention time and spectroscopic data of annotated peaks in *Eschscholzia californica* 70% EtOH extract.

Peak number	Retention time (min)	UV maxima (nm)	ESI MS (+) (m/z)	ESI MS (-) (m/z)	Identification
1	14.4	219, 255, 353	757.1 $[M+H]^+$	755.1 $[M-H]^-$	Rutin rhamnoside
2	16.7	216, 255, 353	611.0 $[M+H]^+$	609.1 $[M-H]^-$	Rutin
3	18.7	204, 254, 353	625.1 $[M+H]^+$	623.1 $[M-H]^-$	Rhamnoside of O-methyl quercetin
4	13.8	* OV	354.0 $[M+H]^+$	-	Protopine
5	14.9	* OV	337.9 $[M+H]^+$	-	Californidine
6	15.5	* OV	324.0 $[M+H]^+$	-	Escholtzine
7	17.3	* OV	340.0 $[M+H]^+$	-	Neocaryachine-7-*O*-methyl ether *N*-metho salt
a	10.6	202, 255, 353	773.0 $[M+H]^+$	771.1 $[M-H]^-$	Unidentified flavonoid
b	18.7	204, 254, 353	625.1 $[M+H]^+$	623.1 $[M-H]^-$	Unidentified flavonoid

* UV spectrum overlapped by coeluting flavonoids.

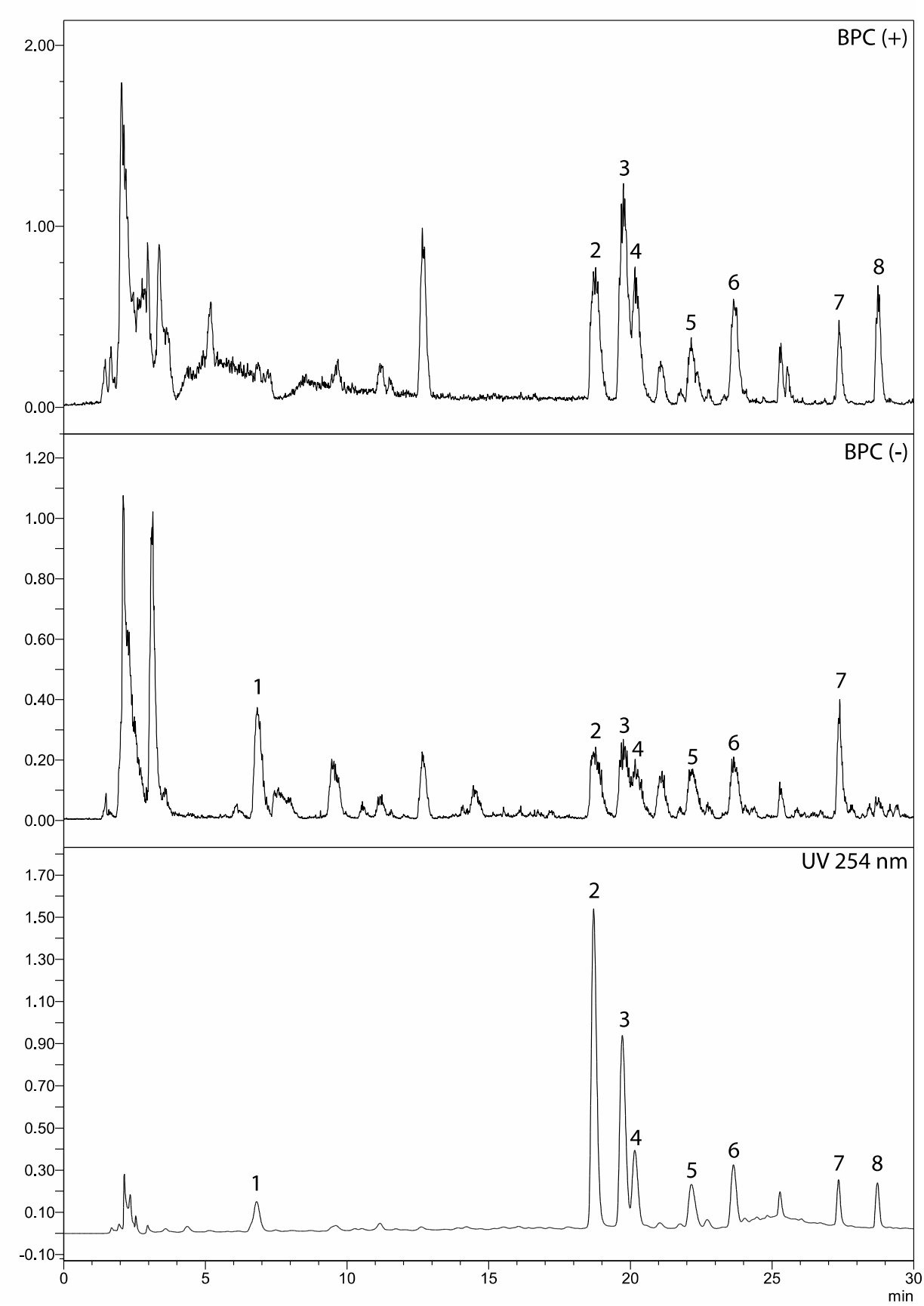

Figure 4S. HPLC-UV-MS analysis of *Hypericum perforatum* 70% EtOH extract. BPC: base peak chromatogram. **1:** chlorogenic acid isomer (tentative assignment), **2:** rutin, **3:** hyperosid, **4:** isoquercitrin, **5:** miquelianin (quercetin-3-*O*-glucuronide), **6:** quercitrin, **7:** quercetin, **8:** biapigenin.

Table 4S. Retention time and spectroscopic data of annotated peaks in *Hypericum perforatum* 70% EtOH extract.

Peak number	Retention time (min)	UV maxima (nm)	ESI MS (+) (m/z)	ESI MS (-) (m/z)	Identification
1	6.8	210, 300, 325	355.0 $[M+H]^+$	353.1 $[M-H]^-$	Chlorogenic acid isomer
2	18.7	221, 255, 351	609.0 $[M+H]^+$	611.0 $[M-H]^-$	Rutin
3	19.7	215, 255, 353	464.9 $[M+H]^+$	463.1 $[M-H]^-$	Hyperosid
4	20.2	201, 255, 353	465.0 $[M+H]^+$	463.1 $[M-H]^-$	Isoquercitrin
5	22.2	201, 255, 353	478.9 $[M+H]^+$	477.0 $[M-H]^-$	Miquelianin
6	23.6	201, 254, 349	449.0 $[M+H]^+$	447.0 $[M-H]^-$	Quercitrin
7	27.4	204, 254, 370	302.9 $[M+H]^+$	301.0 $[M-H]^-$	Quercetin
8	28.7	208, 268, 330	538.9 $[M+H]^+$	537.0 $[M-H]^-$	Biapigenin

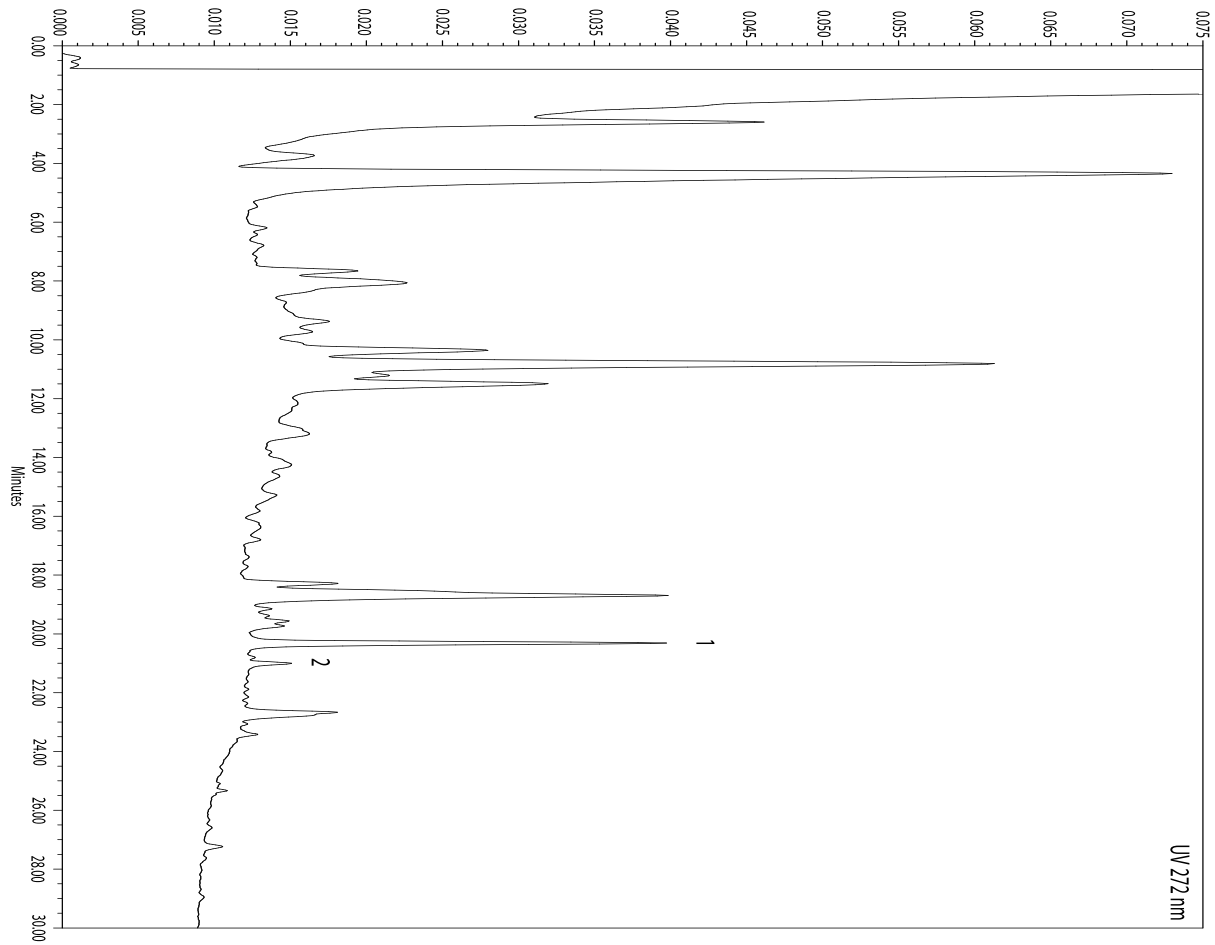

Figure 5S. HPLC-UV analysis of hyperforin and adhyperforin in *Hypericum perforatum* extract. **1:** hyperforin, **2:** adhyperforin.

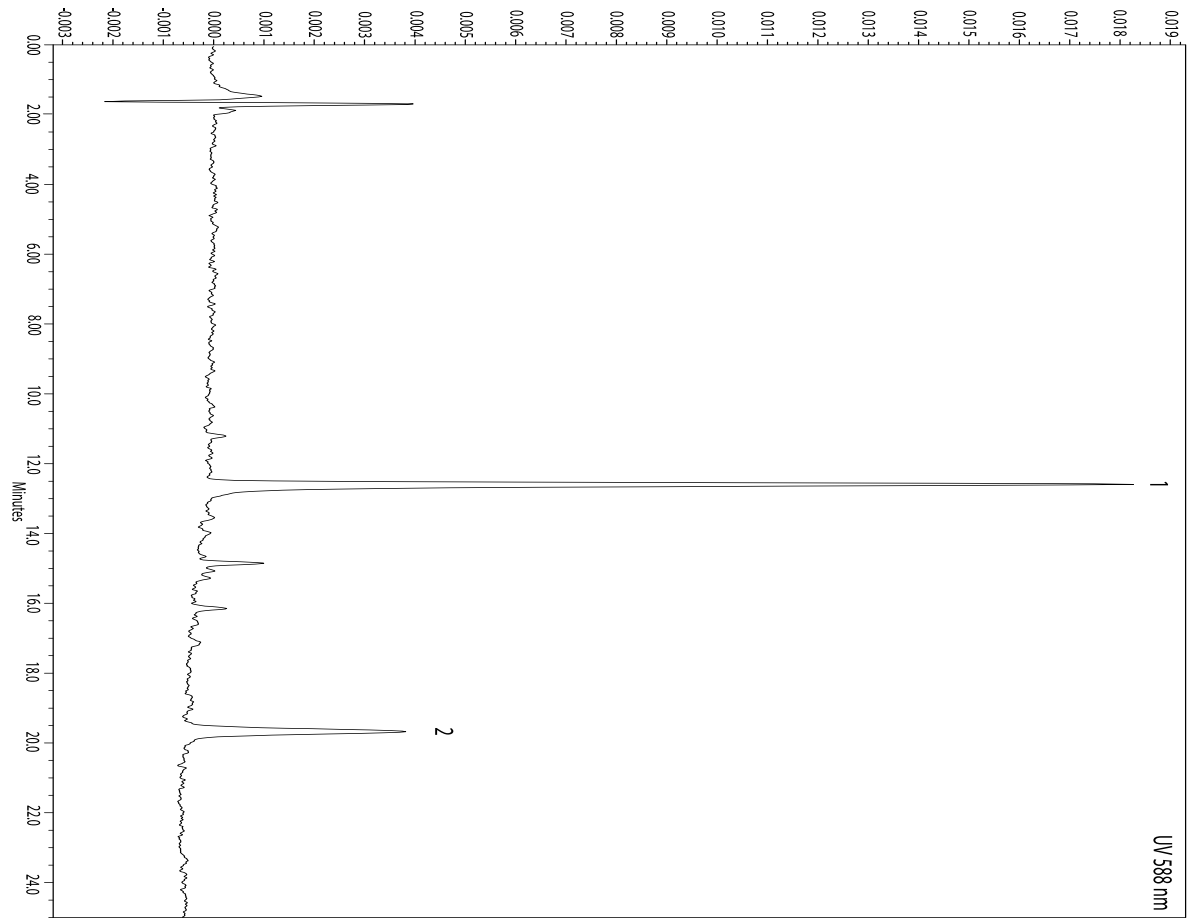

Figure 6S. HPLC-UV analysis of hypericin and pseudohypericin in *Hypericum perforatum* extract. **1:** pseudohypericin, **2:** hypericin.

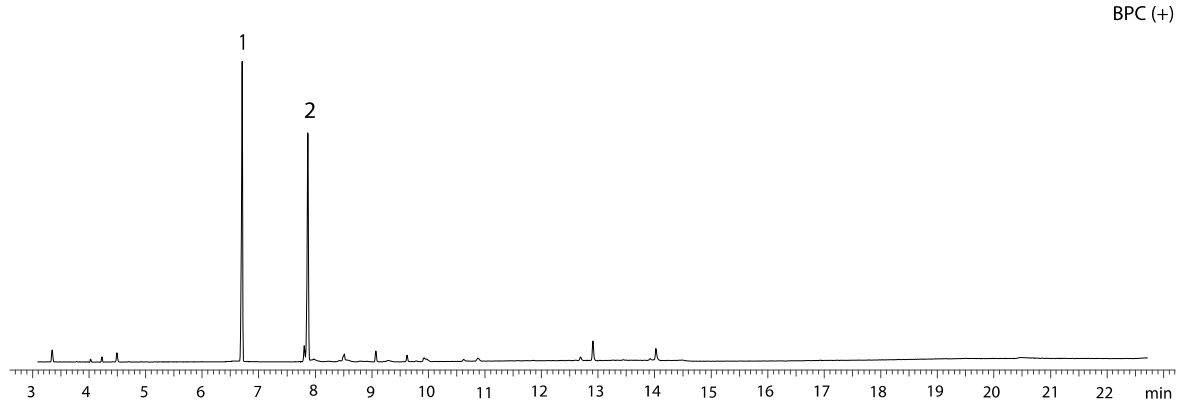

Figure 7S. GC-MS analysis of *Lavandula angustifolia* essential oil. **1:** linalool, **2:** linalyl acetate.

Table 5S. Retention time, peak area and identification of constituents of *Lavendula augustifolia* lavender essential oil.

Peak number	Rentention time (min)	Area (%)	Identification	Spectrum identity (%, NIST)
1	6.71	43.4	Linalool	97
2	7.87	38.6	Linalyl acetate	90

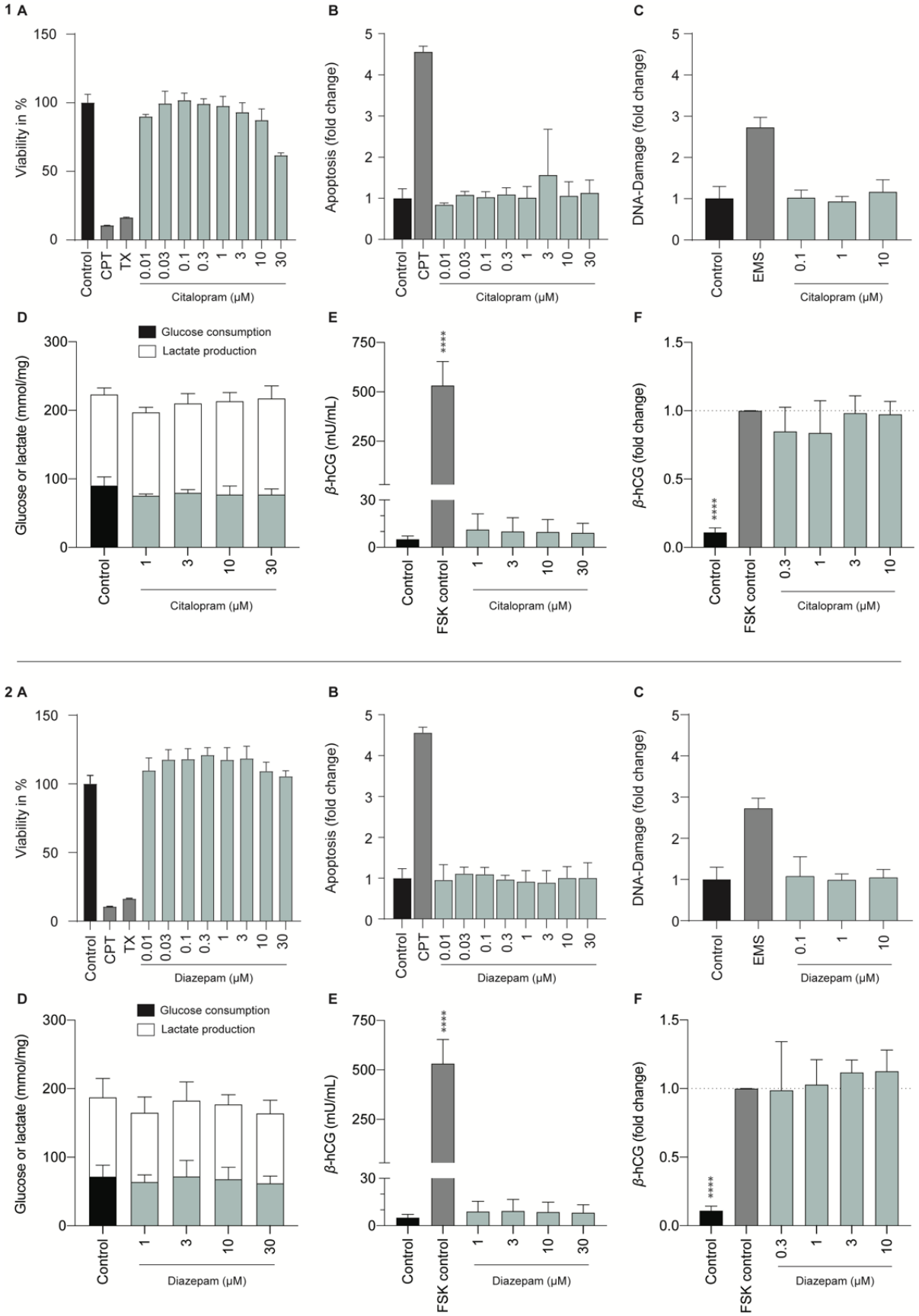

Figure 8S. Effects of different concentrations of citalopram **(1)** and diazepam **(2)** on *in vitro* safety using undifferentiated BeWo b30 cells. All controls consisted of cell culture media containing 0.2% of DMSO. All data were obtained from at least three independent experiments (D-F in triplicate) and are shown as mean ± SD: *$p < 0.05$.

(A) Effects on cell viability after treatment for 72 h as per cent compared to the untreated control; treatments with camptothecin (CPT) and Triton-X-100 (TX) serve as toxicity controls. Results were normalised to untreated control signal = 100%. **(B)** Effects on cell death after treatment for 72 h as fold changes compared to the untreated controls with camptothecin (CPT) for apoptosis. **(C)** Effects on tail DNA after treatment for 3 h as fold changes compared to the untreated control with ethyl methanesulfonate (EMS) as a positive control. **(D)** Effects on glucose consumption and lactate production after treatment for 48 h expressed in mmol and normalised per amount of protein (mg). **(E)** Effects on *β*-hCG secretion (mU/mL) by BeWo b30 cells upon 48 h treatment with increasing concentrations of citalopram **(1)** or diazepam **(2)** and 50 μM FSK as a positive control. (**F)** Effects on FSK-induced differentiation of BeWo b30 cells as fold changes compared to the FSK control. Cells were pre-treated with test compound or cell culture medium for 24 h, before the addition of 5 μM FSK for a further 24 h.

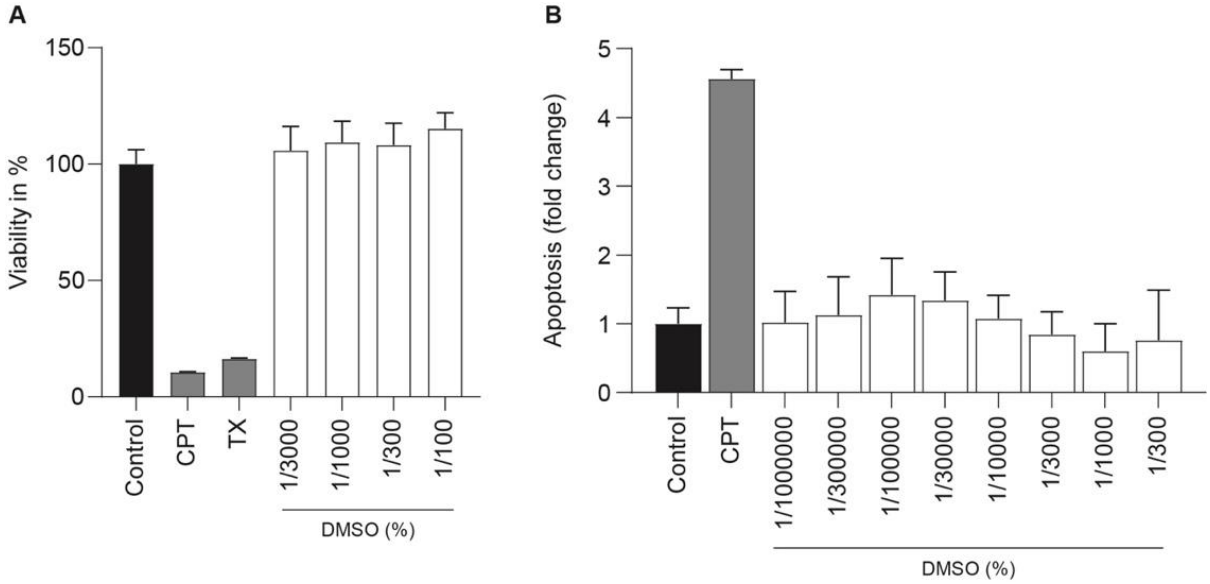

Figure 9S. (A) Cytotoxic effects of DMSO (0.03-1%) on BeWo b30 cells. Viability assays were performed after a 72 h incubation period. Camptothecin (CPT) and Triton-X-100 (TX) were used as toxicity controls. **(B)** Effects of DMSO (0.0001-0.3%) on cell death after treatment for 72 h as fold changes compared to the untreated controls with camptothecin (CPT) for apoptosis. All data are represented as mean ± SD of three independent experiments ($n = 3$; $p^* < 0.05$).

Chapter 3

Chapter 3

Advanced *in vitro* safety assessment of herbal medicines for the treatment of non-psychotic mental disorders in pregnancy

Deborah Spiess, Moritz Winker, Alexandra Dolder Behna, Carsten Gründemann, Ana Paula Simões-Wüst

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When confronted with non-psychotic mental disorders, pregnant women often refrain from using synthetic drugs and resort to herbal medicines such as St. John's wort, California poppy, valerian, lavender, and hops. Nevertheless, these herbal medicines have not yet been officially approved in pregnancy due to lack of safety data. Using a variety of *in vitro* methods (determination of cytotoxicity, apoptosis induction, genotoxicity, effects on metabolic properties, inhibition/induction of differentiation) in a commonly used placental cell line (BeWo b30), we were previously able to show that extracts from these plants are likely to be safe at the usual clinical doses. In the present work, we wanted to extend our safety assessment of these herbal medicines by 1) looking for possible effects on gene expression and 2) using the same *in vitro* methods to characterize effects of selected phytochemicals that might conceivably lead to safety issues. Proteomics results were promising, as none of the five extracts significantly affected protein expression by up- or down-regulation. Protopine (contained in California poppy), valerenic acid (in valerian), and linalool (in lavender) were inconspicuous in all experiments and showed no adverse effects. Hyperforin and hypericin (two constituents of St. John's wort) and valtrate (typical for valerian) were the most obvious phytochemicals with respect to cytotoxic and apoptotic effects. A decrease in cell viability was evident with hypericin (≥ 1 μM) and valtrate (≥ 10 μM), whereas hyperforin (≥ 3 μM), hypericin (30 μM) and valtrate (≥ 10 μM) induced cell apoptosis. None of the tested phytochemicals resulted in genotoxic effects at concentrations of 0.1 and 1 μM and thus are not DNA damaging. No decrease in glucose consumption or lactate production was observed under the influence of the phytochemicals, except for valtrate (at all concentrations). No compound affected cell differentiation, except for hyperforin (≥ 1 μM), which had an inhibitory effect. This study suggests that extracts from St. John's wort, California poppy, valerian, lavender, and hops are likely to be safe during pregnancy. High plasma concentrations of some relevant compounds – hyperforin and hypericin from St. John's wort and valtrate from valerian – deserve special attention, however.

Contributions of Deborah Spiess to this publication: designing and conducting in vitro assays focusing on metabolic activity and placental cell differentiation, interpretation of proteomics data, analysis and interpretation of data, data visualization, writing the first manuscript draft, and manuscript revision

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Advanced *in Vitro* Safety Assessment of Herbal Medicines for the Treatment of Non-Psychotic Mental Disorders in Pregnancy

Deborah Spiess[1], *Moritz Winker*[2,3], *Alexandra Dolder Behna*[1], *Carsten Gründemann*[2,3] *and Ana Paula Simões-Wüst*[1]*

[1]Department of Obstetrics, University Hospital Zurich, University of Zurich, Zurich, Switzerland, [2]Center for Complementary Medicine, Faculty of Medicine, University of Freiburg, Freiburg, Germany, [3]Translational Complementary Medicine, Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland

When confronted with non-psychotic mental disorders, pregnant women often refrain from using synthetic drugs and resort to herbal medicines such as St. John's wort, California poppy, valerian, lavender, and hops. Nevertheless, these herbal medicines have not yet been officially approved in pregnancy due to lack of safety data. Using a variety of *in vitro* methods (determination of cytotoxicity, apoptosis induction, genotoxicity, effects on metabolic properties, and inhibition/induction of differentiation) in a commonly used placental cell line (BeWo b30), we were previously able to show that extracts from these plants are likely to be safe at the usual clinical doses. In the present work, we wanted to extend our safety assessment of these herbal medicines by 1) looking for possible effects on gene expression and 2) using the same *in vitro* methods to characterize effects of selected phytochemicals that might conceivably lead to safety issues. Proteomics results were promising, as none of the five extracts significantly affected protein expression by up- or down-regulation. Protopine (contained in California poppy), valerenic acid (in valerian), and linalool (in lavender) were inconspicuous in all experiments and showed no adverse effects. Hyperforin and hypericin (two constituents of St. John's wort) and valtrate (typical for valerian) were the most obvious phytochemicals with respect to cytotoxic and apoptotic effects. A decrease in cell viability was evident with hypericin (≥1 μM) and valtrate (≥10 μM), whereas hyperforin (≥3 μM), hypericin (30 μM) and valtrate (≥10 μM) induced cell apoptosis. None of the tested phytochemicals resulted in genotoxic effects at concentrations of 0.1 and 1 μM and thus are not DNA damaging. No decrease in glucose consumption or lactate production was observed under the influence of the phytochemicals, except for valtrate (at all concentrations). No compound affected cell differentiation, except for hyperforin (≥1 μM), which had an inhibitory effect. This study suggests that extracts from St. John's wort, California poppy, valerian, lavender, and hops are likely to be safe during pregnancy. High plasma concentrations of some relevant

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***Correspondence:**
Ana Paula Simões-Wüst
anapaula.simoes-wuest@usz.ch

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Abbreviations: β-hCG, beta-human chorionic gonadotropin; CPT, camptothecin; EMS, ethyl methanesulfonate; FC, fold change; FSK, forskolin; NMD, non-psychotic mental disorder; TX, Triton-X-100.

compounds—hyperforin and hypericin from St. John's wort and valtrate from valerian—deserve special attention, however.

Keywords: mental health disorders, pregnancy, safety, *Hypericum perforatum*, *Eschscholzia californica*, *Valeriana officinalis*, *Lavandula angustifolia*, *Humulus lupulus*

1 INTRODUCTION

Non-psychotic mental disorders (NMDs) are a common problem during pregnancy. One multinational study found that on average 28.9% of pregnant women used herbal medicines during pregnancy, although there were significant differences between regions/countries, and that the prevalence in Switzerland was thus very high at 40.6% (Kennedy et al., 2013). In a recent survey we conducted in Switzerland, 51.3% of participants reported a mental symptom during pregnancy (such as insomnia, anxiety, and depressive mood). In addition, 13.2% and 4.0% suffered from acute and chronic mental disorders, respectively. Interestingly, only a few participants used antidepressants (e.g., sertraline, citalopram) and none mentioned (or at least indicated) the use of sedatives/anxiolytics (e.g., lorazepam, diazepam) during pregnancy. This suggests that pregnant women refrain from using synthetic psychoactive medication and prefer resorting to herbal medicines for mild NMD treatment (Gantner et al., 2021). Such behavior is well justified, as psychotropic drugs can lead to side effects for both the mother and the fetus or newborn (Howard et al., 2014; Stewart and Vigod, 2020).

Herbal candidates for the treatment of NMDs in pregnancy include St. John's wort (*Hypericum perforatum* L.), California poppy (*Eschscholzia californica* Cham.), valerian (*Valeriana officinalis* L.), hops (*Humulus lupulus* L.), and lavender (*Lavandula angustifolia* Mill.). St. John's wort is a herbal alternative to synthetic antidepressants in the treatment of mild to moderate depression (Wurglics and Schubert-Zsilavecz, 2006), which does not differ in efficacy from selective serotonin reuptake inhibitors (Cui and Zheng, 2016). California poppy has a long tradition of use in aiding sleep and relieving mild symptoms of mental stress (European Medicines Agency, 2015a). There are currently a variety of California poppy products on the market that are approved as phytomedicines (contain either plant extract or powdered drug), but also various dietary supplements (European Medicines Agency, 2015b; Medicinal product information, 2022). To avoid resorting to synthetic medications for sleep disorders, a combination of valerian and hops offers an alternative treatment. Several randomized trials conclude that the valerian-hops combination mentioned has (modest) hypnotic effects and significantly reduces sleep latency (Morin et al., 2005; Koetter et al., 2007; Dimpfel and Suter, 2008). Constituents of both extracts complement each other: some compounds of valerian act like endogenous adenosine and increase sleepiness, while a few phytochemicals of hops act like endogenous melatonin and support rhythmicity (Brattström, 2007). Although there are a variety of registered valerian-hops preparations on the Swiss market, there are valerian—but not hops—monopreparations (Medicinal product information search platform, 2022). Lavender essential oil is best known for its calming, sedative and anxiolytic effects and is therefore popular in the treatment of restlessness, sleep disorders, and anxiety (Schilcher et al., 2016). The efficacy and safety of a patented essential oil produced from *Lavandula angustifolia* flowers for oral administration has been demonstrated in adult patients with generalized anxiety disorder in several trials (Woelk and Schläfke, 2010; Kasper et al., 2014).

The evidence for the safety of the herbal medicines mentioned above in pregnancy is, on the other hand, very limited or non-existent. The Committee on Herbal Medicinal Products of the European Medicine Agency does not recommend the use of these herbal medicines during pregnancy due to insufficient safety data (European Medicines Agency, 2009; European Medicines Agency, 2012; European Medicines Agency, 2014; European Medicines Agency, 2015a; European Medicines Agency, 2016). In our survey, 3.5% of pregnant women reported taking St. John's wort, 5.8% used valerian, lavender was used by 16.2% of women, and hops by 2.3% (Gantner et al., 2021).

In examining the phytochemical composition of the selected herbal candidates for treatments in pregnancy, several compounds stand out that are highly biologically active, found in various species of interest, and/or very abundant and thus might potentially cause problems from a safety perspective. In our opinion, these include hyperforin and hypericin, which are the most abundant representatives of phloroglucinol derivatives (0.2–0.4% of total dry weight of the herb) and naphthodianthrones (0.1–0.3% of total dry weight of the herb) in St. John's wort (Blaschek, 2016). Also, protopine deserves particular attention as one of the major isoquinoline alkaloids of California poppy (Tomè et al., 1999; Fedurco et al., 2015), which is also present in several other herbal medicines (Guinaudeau and Shamma, 1982). Furthermore, we chose valerenic acid and valtrate, which are important representatives of the sesquiterpenes and the valepotriates present in valerian root (Blaschek, 2016). Finally, linalool was considered, since it corresponds to approximately one quarter (26.1%) of lavender oil and is often one of the major components in essential oils produced by a variety of plants such as hops, coriander, and star anise (Karabín et al., 2016; Schilcher et al., 2016). Linalool was identified as the major pharmacologically active constituent involved in the anti-anxiety effect of lavender essential oil (Umezu et al., 2006).

In our previous work, we conducted an initial *in vitro* assessment of the safety profile of extracts from St. John´s wort, California poppy, valerian, lavender, and hops. In concentrations up to 30 µg/ml, they did not possess any cytotoxic or genotoxic potential and did not compromise human placental cell viability, metabolic activity or differentiation (Spiess et al., 2021). In a next step, our research

goals were to 1) look for possible effects of whole extracts on gene expression and 2) use the same variety of methods as before to characterize effects of particularly relevant phytochemicals from these extracts. For the above-mentioned reasons, the latter include hypericin, hyperforin, protopine, valerenic acid, valtrate, and linalool.

2 MATERIALS AND METHODS

2.1 Cell Culture

The BeWo b30 cell line was provided by Dr. Tina Buerki-Thurnherr (Empa - Swiss Federal Laboratories for Materials Science and Technology, St. Gallen, Switzerland), with permission from Dr. Alan L. Schwartz (Washington University School of Medicine, MO, United States). As described in a previous study (Spiess et al., 2021), cells were cultivated in F-12K Nut Mix supplemented with 10% heat inactivated FBS, antibiotics (100 U/ml penicillin, 100 µg/ml streptavidin) and 2 mM L-glutamine (all from Gibco), at 37°C and in 5% CO_2 humidified atmosphere.

2.2 Cell Treatments

All plant material was of Ph. Eur. grade. *V. officinalis* roots, *H. lupulus* flowers and *H. perforatum* herb were purchased from Dixa (lot numbers 180084, 191241, 192140, respectively). *E. californica* herb was obtained from Galke (lot number 811502). Voucher specimens (numbers 1029, 1167, 1166 and 1234, respectively) have been deposited at the Division of Pharmaceutical Biology, University of Basel. The powdered plant material was extracted with 70% EtOH by pressurized liquid extraction in a Dionex ASE 200 Accelerated Solvent Extractor. Three cycles of extraction of 5 min each were performed at a temperature of 70°C and a pressure of 120 bar. *L. angustifolia* essential oil Ph. Eur. was purchased from Hänseler (lot number 2018.01.0274). See Supporting Information of our previous work (Spiess et al., 2021) for characterization of all hydroalcoholic extracts and of *L. angustifolia* essential oil. The following phytochemicals were obtained from commercial sources: hyperforin, hyperforin dicyclohexylammonium salt and linalool (Sigma-Aldrich), protopine and valerenic acid (Extrasynthese), and valtrate (Toronto Research Chemicals). Dry herbal extracts and pure phytochemicals were dissolved in sterile DMSO (Sigma-Aldrich) and stored at -80°C. BeWo b30 cells were treated with increasing concentrations of phytochemicals (up to 30 µM, a concentration higher than all reported achieved plasma concentrations; compare with discussion) in culture medium. Control cells were exposed to a final concentration of ≤0.3% DMSO so as not to decrease cell viability. The biochemical and morphological differentiation of BeWo b30 cells was stimulated with 5 µM forskolin (FSK; Lucerna-Chem; dilution from a 30 mM stock solution prepared in DMSO). Due to light sensitivity and to avoid phototoxicity under treatment with St. John's wort extract, hyperforin and hypericin, the relevant experiments were performed with as little light as possible.

2.3 Protein Analysis

2.3.1 Sample Preparation

BeWo b30 cells were seeded into transparent T25 culture flasks at a density of 776′400 cells/6,000 µl/flask. After overnight incubation, they were exposed to 30 µg/ml of plant extract (*H. perforatum*, *E. californica*, *V. officinalis*, *L. angustifolia* essential oil, *H. lupulus*) or untreated control (0.06% DMSO) for 48 h. Cell culture supernatants were then discarded and attached cells were rinsed with PBS and then detached by adding a 0.25% trypsin-EDTA solution, following 1–2 min incubation (37°C). The trypsinization process was stopped by the addition of cell culture growth medium and the flasks were rinsed carefully. The cell suspensions were centrifuged (at 1,300 rpm at 23°C for 5 min). The cell pellets were washed a total of three times (by aspirating the supernatant and resuspending the cell pellet in 10 ml PBS). The cells were then counted and 1′000′000 cells per condition were centrifuged. The corresponding cell pellets were immediately snap-frozen in liquid nitrogen (without buffer) and stored at −80°C for subsequent analysis of proteins.

For each sample, proteins were extracted using a tissue homogenizer (TissueLyser II, QUIAGEN) and digested by using a commercial iST Kit (PreOmics). Briefly, 150 µl of 'Lyse' buffer and around 150 mg of glass beads (425–500 µm, Sigma) were added to each cell pellet. After 2 cycles of protein extraction (2 min each, 30 Hz) and incubation for 10 min at 95°C, the solubility of the extracted proteins was enhanced by processing the samples with High Intensity Focused Ultrasound for 1 min, setting the ultrasonic amplitude to 85% followed by additional incubation for 10 min at 95°C. After centrifugation for 10 min at 14′000 g the supernatant was used for further processing. The proteins (50 µg in each case) were digested on the membrane by adding 50 µl of the 'Digest' solution. After 60 min of incubation at 37°C the digestion was stopped with 100 µl of, Stop, solution. The samples were then centrifuged, and the supernatant was transferred to the cartridge. The solutions in the cartridge were removed by centrifugation at 3,800 *g*, while the peptides were retained by the iST-filter. Finally, the peptides were washed, eluted, dried and re-solubilized in 20 µl of injection buffer (3% acetonitrile, 0.1% formic acid). Prior to LC-MS analysis, the peptide concentrations were estimated by means of a NanoDrop spectrophotometer, and the injection amounts normalized to an absorbance of 0.3 at 280 nm.

2.3.2 Liquid Chromatography-Mass Spectrometry Analysis

Mass spectrometry analysis was performed on an Orbitrap Fusion Lumos (Thermo Scientific) equipped with a Digital PicoView source (New Objective) and coupled to an M-Class UPLC (Waters). Solvent composition at the two channels was 0.1% formic acid for channel A and 0.1% formic acid, 99.9% acetonitrile for channel B. For each sample, 1 µl of diluted peptides were loaded on a commercial MZ Symmetry C18 Trap Column (100Å, 5 µm, 180 µm × 20 mm, Waters) followed by a nanoEase MZ C18 HSS T3 Column (100 Å, 1.8 µm, 75 µm × 250 mm, Waters). The peptides were eluted at a flow rate of 300 L/min by a gradient from 5 to 22% B in 77 min, 32% B in 10 min and 95% B for 10 min. Samples were acquired in a randomized order.

The mass spectrometer was operated in data-dependent mode, acquiring a full-scan MS spectra (300–1,500 *m/z*) at a resolution of 120′000 at 200 *m/z* after accumulation to a target value of 500′000. Data-dependent MS/MS were recorded in the linear ion trap using quadrupole isolation with a window of 0.8 Da and HCD fragmentation with 35% fragmentation energy. The ion trap was operated in rapid scan mode with a target value of 10′000 and a maximum injection time of 50 ms. Only precursors with an intensity above 5′000 were selected for MS/MS and the maximum cycle time was set to 3 s. Charge state screening was enabled. Singly, unassigned and charge states higher than seven were rejected. Precursor masses previously selected for MS/MS measurement were excluded from further selection for 20 s, and the exclusion window was set at 10 ppm. The samples were acquired using internal lock mass calibration on *m/z* 371.1012 and 445.1200. The mass spectrometry proteomics data were handled using the local laboratory information management system (Türker et al., 2010) and all relevant data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD031765.

2.3.3 Protein Identification and Label Free Protein Quantification

The acquired raw MS data were processed by MaxQuant (version 1.6.2.3), followed by protein identification using the integrated Andromeda search engine (Cox and Mann, 2008). One separate MaxQuant experiment was set up for each set of sample (B015, B020, B035, and HMEV21). Spectra were searched against a swissprot canonical *Homo sapiens* proteome (taxonomy 9,606, version from 2019 to 07-09), concatenated to its reversed decoyed fasta database and common protein contaminants. Carbamidomethylation of cysteine was set as fixed modification, while methionine oxidation and N-terminal protein acetylation were set as variable. Enzyme specificity was set to trypsin/P allowing a minimal peptide length of 7 amino acids and a maximum of two missed-cleavages. MaxQuant Orbitrap default search settings were used. The maximum false discovery rate was set to 0.01 for peptides and 0.05 for proteins. Label free quantification was enabled and a 2 min window for match between runs was applied. In the MaxQuant experimental design template, each file is kept separate in the experimental design to obtain individual quantitative values. The MaxQuant results were loaded into Scaffold (Proteome Software Inc.) to validate the peptide and protein identifications. Only proteins identified with at least 2 peptides were considered for follow up analysis. The statistical significance threshold was set to an adjusted p-value ≤ 0.2, and a $\log_2 FC > \pm 1$.

2.4 Functional Assays

The same plethora of functional assays was used as in our previous work with herbal extracts (Spiess et al., 2021), as briefly described in the following.

2.4.1 Viability Assay

The *in vitro* cytotoxicity of the different concentrations of phytochemicals (0.01, 0.03, 0.1, 0.3, 1, 3, 10, and 30 μM) was tested using a WST-1 viability assay. BeWo b30 cells were seeded in 96-well flat-bottom plates with a density of 2×10^4 cells/100 μl/well on the day before exposure to the phytochemical dilutions in fresh culture medium. Camptothecin (CPT, 300 μM; apoptosis control; Tocris Bioscience) or 0.5% Triton-X-100 (TX, necrosis control; Sigma-Aldrich) served as positive controls. After 72 h of incubation, culture supernatant was aspirated and replaced by medium without phenol red, and 5 μl Cell Proliferation Reagent WST-1 (Roche) was added. A spectrophotometric measurement was taken (450 nm) after 75 min of incubation, using a plate reader (Tecan Reader Infinite M 200).

2.4.2 Apoptosis Assay

BeWo b30 cells were subjected to the same treatment described for the WST-1 assay to assess the level of apoptosis after application of the test substances. After 72 h of incubation, the cells were washed with PBS and detached using Accutase (Sigma-Aldrich). All liquids were pooled and centrifuged for 5 min (300 g), followed by an AnnexinV-FITC (eBioscience) staining, which was performed according to the manufacturer's instructions. A flow cytometric readout was obtained and analyzed using appropriate software (BD FACScalibur, BD Biosciences, FlowJo Software).

2.4.3 Comet Assay

A comet assay was used to examine the genotoxic potential of the selected phytochemicals. Microscopic slides were coated with 1% normal-melting agarose in PBS (SERVA Electrophoresis GmbH) beforehand. Cells were seeded with a density of 4×10^5 cells/100 μl/well on the day before exposure. Different concentrations of phytochemicals (0.1, 1, and 10 μM) or 3 mM ethyl methanesulfonate (EMS, positive control; Sigma-Aldrich) were added for 3 h. A slightly boiling 0.7% normal-melting agarose solution (200 μl) was then applied to the precoated slides. The cells were once washed with PBS, dissolved using Accutase (Sigma-Aldrich) and resuspended in 30 μl complete medium. The cells were then gently mixed with 90 μl 0.7% low-melting agarose (SERVA Electrophoresis GmbH) solution (rapidly heated to 100°C and kept at 38°C prior to use) and added as a final layer to the slides. Electrophoresis was performed at 25 V/300 mA for 20 min. The slides were finally washed with ddH_2O and PBS and fixed with 99% EtOH. Fixed samples were stained with ethidium bromide solution (5 μg/ml; Carl Roth GmbH), and images were taken for later analysis with CometScore software (version 2.0.038 for Windows; TriTek Corp., United States).

2.4.4 Glucose and Lactate Concentration Measurements

Cells were seeded into transparent 24-well plates at a density of 2.5×10^4 cells/1,000 μl/well. After overnight incubation, they were exposed to different concentrations (1, 3, 10, and 30 μM) of phytochemicals or untreated control (0.2% DMSO) for 48 h. Cell culture supernatants and pellets were collected and immediately frozen at −80°C for subsequent analysis of the metabolic parameters (glucose/lactate) and protein concentrations, respectively. Glucose and lactate were determined using an automated blood gas analyzer (ABL800 Flex, Radiometer Medical ApS). Protein concentrations were determined using

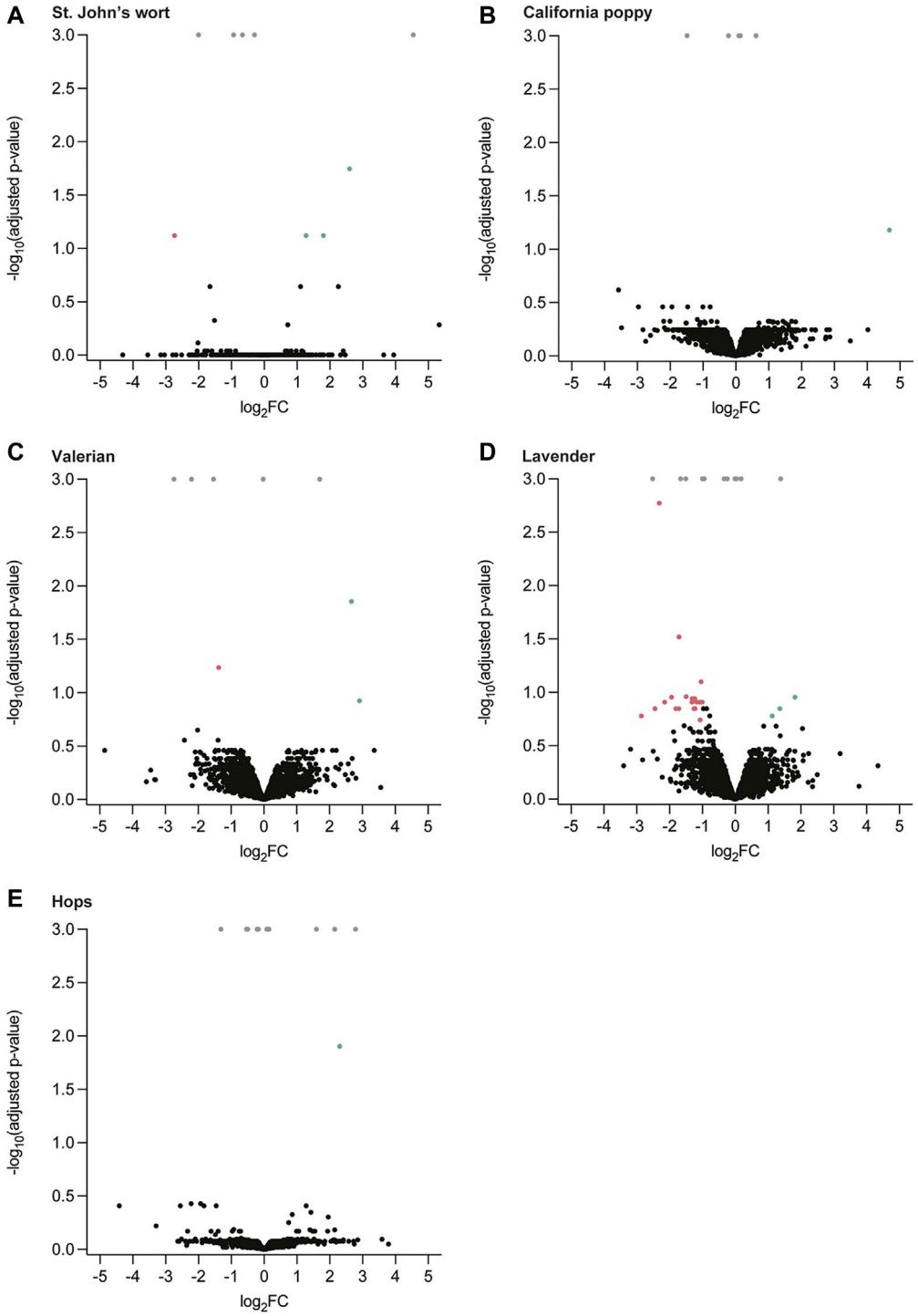

FIGURE 1 | Label-free quantification of proteins of treated (herbal extracts) and untreated (0.06% DMSO) BeWo b30 cells for a period of 48 h. Volcano plots representing identified proteins as $\log_2$ fold change (FC) ratio of protein intensity (treated/untreated), plotted against the significance as a function of negative $\log_{10}$ (adjusted *p*-value). Significantly enriched proteins (adjusted *p*-value ≤ 0.02) are colored accordingly (● $\log_2$FC > 1, ● $\log_2$FC > −1). Insignificantly different proteins are marked in black. Proteins that were only identified in either the treated or untreated sample are displayed in gray (adjusted *p*-value = 0.001). **(A)** St. John's wort, **(B)** California poppy, **(C)** valerian, **(D)** lavender, and **(E)** hops.

the bicinchoninic acid protein assay kit (Pierce) with BSA (Thermo Scientific) as reference standard.

2.4.5 Placental Cell Differentiation and β-hCG Production

To induce differentiation, cells were seeded into 24-well plates (2.5×10^4 cells/1,000 µl/well) on the day before exposure to different concentrations (1, 3, 10 and 30 µM) of phytochemicals, control (0.2% DMSO) or FSK control (5 µM) for 48 h. To test the ability to inhibit differentiation, cells were seeded into 96-well flat-bottom plates (1×10^4 cells/100 µl/well). After an overnight incubation, the cells were incubated with different concentrations of phytochemicals (serial dilution below apoptotic concentrations based on preliminary results), control (0.2% DMSO) or FSK control (5 µM) for 24 h. Afterwards all cells (except control) were differentiated with FSK (5 µM) for another 24 h. Analysis of β-hCG concentrations—a marker of placenta cell differentiation—was performed by standard ELISA using cell culture supernatants.

2.5 Linalool Analysis

GC/MS was performed on a Hewlett-Packard GC/MS system (Agilent G1503A) equipped with a 5,973 Mass Selective Detector and a 59864B Ionization Gauge Controller (Agilent Technologies). A J&W DM-225 GC column (30 m; i.d.: 0.25 mm; film thickness: 0.25 µm; Agilent Technologies) was used. Injector temperature was 260°C. Helium (0.7 ml/min) was used as a carrier gas. Transfer line temperature was 240°C. The following temperature program was applied: 60°C hold for 1 min, increase to 240°C at 10°C/min followed by 5 min at 240°C. EI ionization was in positive ion mode (electron energy: 2040 V; multiple ion detection modus at *m/z* 93, 105, 121, and 161). Data acquisition was performed by MSD ChemStation D.03 software (Agilent Technologies).

2.6 Statistical Data Analysis

Statistical data analyses were performed using GraphPad Prism (version 9.2.0 for macOS; GraphPad Software). The Shapiro-Wilk test was used to check for normal distribution. Multiple group comparisons were performed using the Brown-Forsythe and Welch ANOVA tests, followed by the Dunnett's T3 multiple comparisons posthoc test (with individual variances computed for each comparison). Probability values $^*p \leq 0.05$ were considered statistically significant. The asterisks represent significant differences from the control group ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$). All results are expressed as means ± SD of at least three independent experiments.

3 RESULTS

3.1 Protein analysis

The influence of hydroalcoholic herbal extracts of St. John's wort, California poppy, valerian, and hops, as well as lavender essential oil on the proteome of the placental cell line BeWo b30 was assessed (for their phytochemical characterization, see Supporting Information in (Spiess et al., 2021)). Label-free protein quantification showed that none of the five extracts considerably influenced the expression of proteins by up- or downregulation (**Figure 1**). From approximately 4,000 proteins identified in each case, only a few exceptional proteins were significantly altered after incubation with the herbal extracts of St. John's wort (4/4,060; RN149, TAP26, MBOA7, and PPR21), California poppy (1/3,353; ANR35), valerian (3/3,423; COPRS, AN32B, and FLOT2), hops (1/3,387; NAGAB), and lavender (24/3,999; CALB2, SPTB1, GEMI, BGLR, UBQL2, RIOX2, STT3B, STT3A, SET, PEBB, SC11A, TTC9C, TBA1B, CCS, CACO2, GALK1, TLE1, TYSY, COQ9, RL36, TF2AA, URM1, S61A1, and EIF3D) were significantly altered (see **Supplementary Table S1** for additional information and their functions). Some proteins were only identified in either the treated (herbal extract) or untreated (0.06% DMSO) sample (adjusted *p*-value = 0.001; see **Supplementary Table S2–S6** for additional information). Most importantly, no enrichments of biological processes, molecular functions, cellular components, or protein classes and pathways were found when submitting the list of differentially regulated or only in one condition identified proteins to a tool for Gene-Set Enrichment analysis. The amounts of most of the proteins were similar in the absence and presence of the extracts.

3.2 Functional Assays

To perform an in-depth analysis of the safety of the herbal medicines investigated, phytochemicals that might conceivably cause issues from a safety point of view were chosen. With these individual compounds, we performed various *in vitro* assessments in BeWo cells, starting with effects on cell viability (**Figure 2**). Hyperforin, which is present in St. John's wort extract, did not lead to pronounced cytotoxic effects in a concentration range of 0.01–30 µM. However, for hypericin, which is also a major component of the same extract, full viability was only maintained up to a concentration of 0.3 µM, since at 1, 3, 10, and 30 µM it was strongly reduced. Protopine, a representative of California poppy, did not reduce cell viability from a concentration range of 0.01–10 µM; a reduction in cell viability was observed at 30 µM only. Valerenic acid, which is present in valerian extract, showed no effect on cell viability up to high concentrations of 30 µM. In contrast, valtrate (also present in valerian extract) decreased viability at 10 and 30 µM by 83% and 89%, respectively. Linalool (contained in lavender essential oil) showed no cytotoxicity after 72 h of incubation at concentrations of up to 30 µM. Since linalool is highly volatile, we investigated whether (calculated) concentration was retained during diverse experimental conditions at 37°C (**Supplementary Table S7**). Results show that after 3 h, 60–85% of the original linalool was still present in the cell culture medium, whereas after 48 h, this corresponded to 36–44%.

Protopine, valerenic acid, and linalool did not induce apoptosis at concentrations ranging from 0.01 to 30 µM (**Figure 3**). However, hyperforin at concentrations of 3 µM and above, and valtrate at concentrations of 10 and 30 µM revealed increased apoptotic levels compared to control. Hypericin showed a clear-cut even if not significant increase at 1 µM. At higher concentrations, and also in accordance with the results of the viability assay, the number of total detected cells was

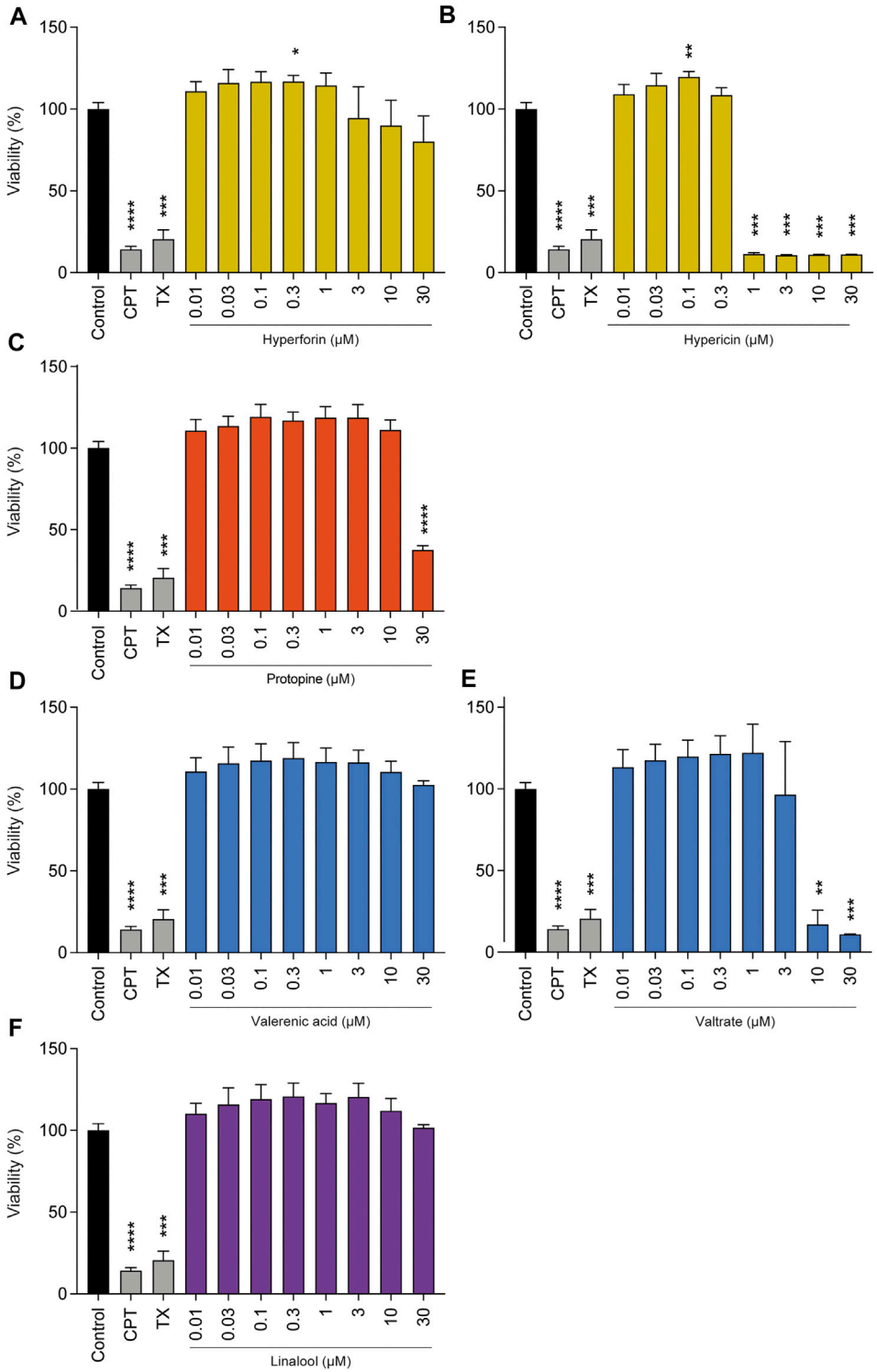

FIGURE 2 | Effects of phytochemicals on cell viability of undifferentiated BeWo b30 cells after 72 h of treatment. Among the phytochemicals present in St. John's wort, such as hyperforin **(A)** and hypericin **(B)**, only the latter resulted in a significant reduction in cell viability at concentrations of 1, 3, 10, and 30 μM. Protopine **(C)** (present in California poppy) reduced cell viability by 62.4% at a concentration of 30 μM. Of the phytochemicals present in valerian, such as valerenic acid **(D)** and valtrate **(E)**, only the latter resulted in a significant reduction in cell viability at concentrations of 10 and 30 μM. Linalool **(F)** (ingredient of lavender oil) did not show any significant effect in a concentration range from 0.01 up to 30 μM. The effects are shown as fold change compared to the untreated control. Treatments with camptothecin (CPT, 300 μM) and Triton-X-100 (TX, 0.5%) served as toxicity controls. Results were normalized to untreated control signal = 100% (n = 3).

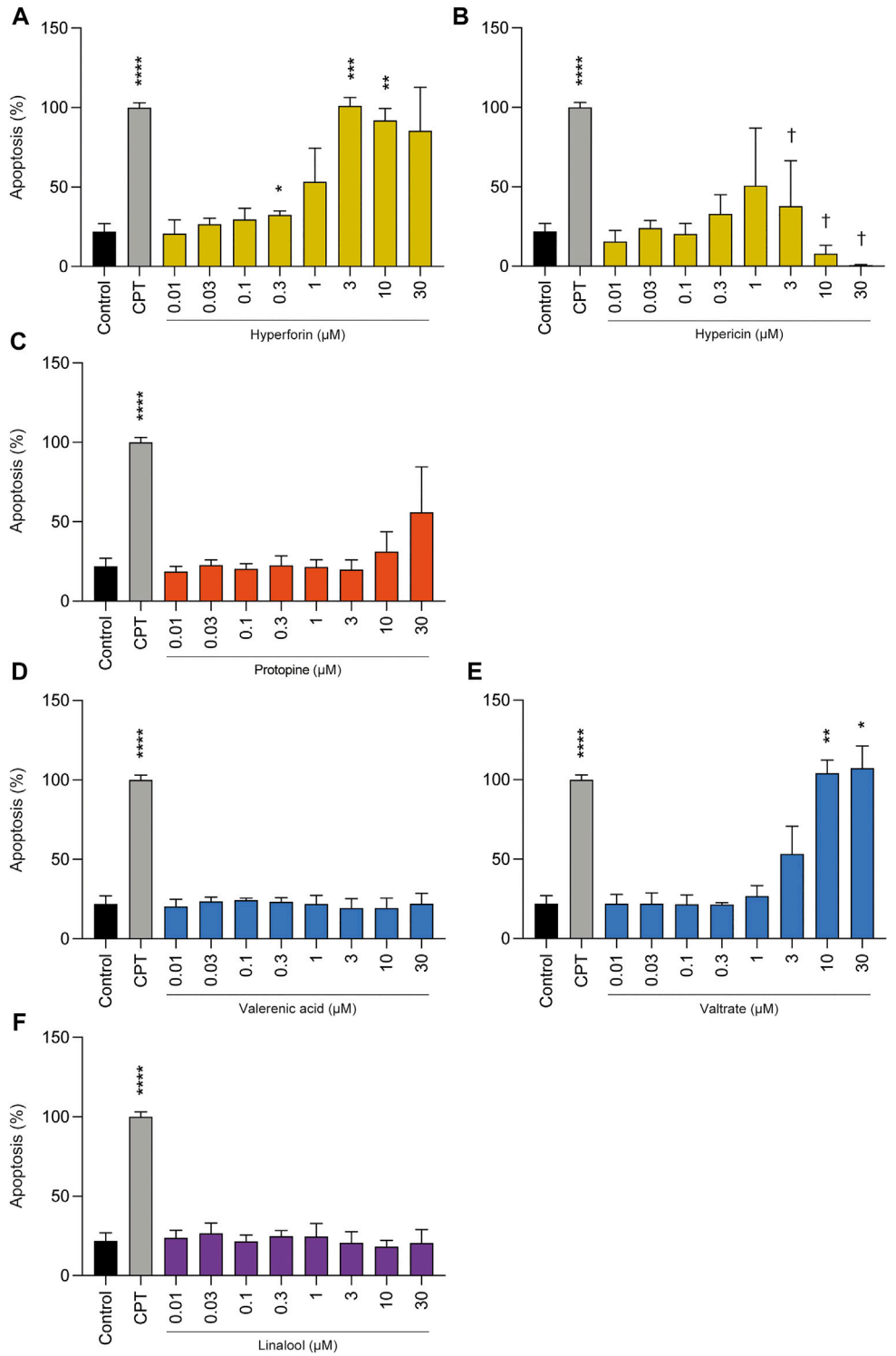

FIGURE 3 | Effects of phytochemicals on cell death of undifferentiated BeWo b30 cells after treatment for 72 h: **(A)** hyperforin, **(B)** hypericin, **(C)** protopine, **(D)** valerenic acid, **(E)** valtrate, and **(F)** linalool. Apoptosis only significantly increased for the highest concentrations of hyperforin (≥3 μM), and valtrate (≥10 μM). Hypericin showed a non-significant increase at 1 μM followed by a significant decrease up to the highest concentration due to an overall decrease in detected cells († cell detection was limited due to advanced degradation). Results were normalized to camptothecin (CPT, 300 μM) = 100% (*n* = 3), which was used as positive control for apoptosis.

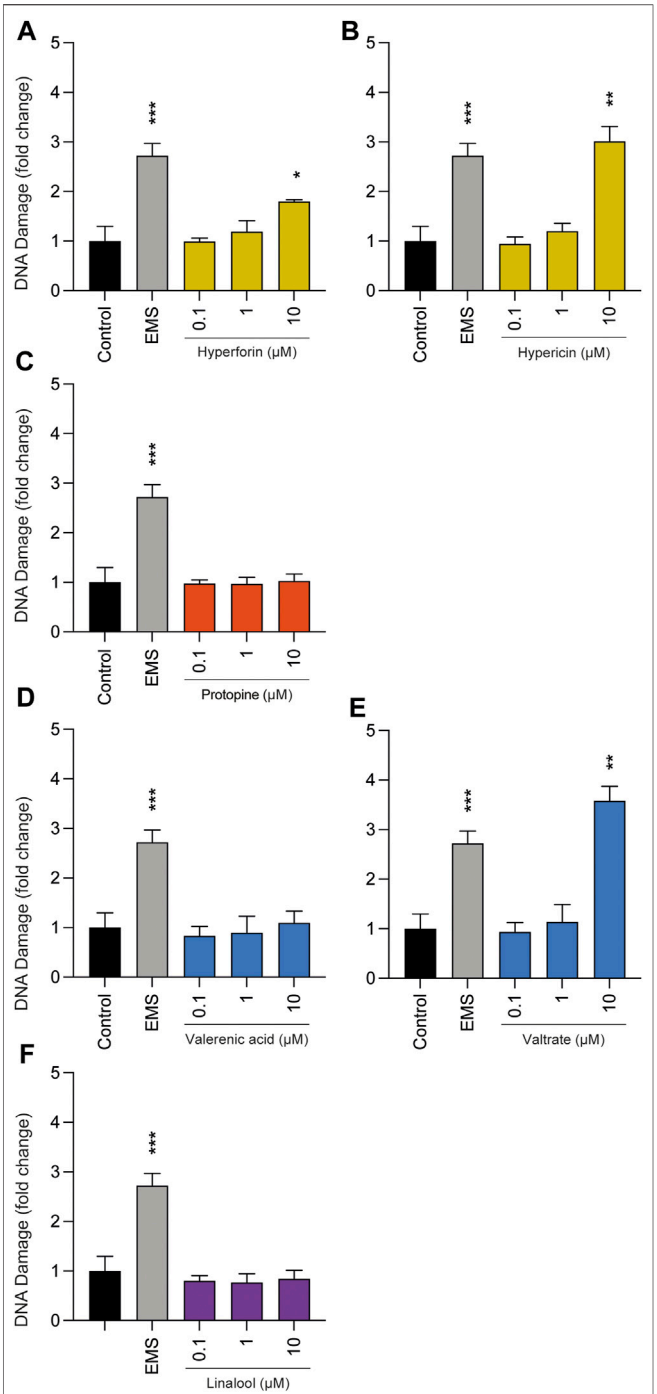

FIGURE 4 | Effects of phytochemicals **(A)** hyperforin, **(B)** hypericin, **(C)** protopine, **(D)** valerenic acid, **(E)** valtrate, and **(F)** linalool on tail DNA in undifferentiated BeWo b30 cells after exposure for 3 h. No significant genotoxic effects were observed at concentrations of 0.1 and 1 μM. Only the highest concentrations (10 μM) of hyperforin, hypericin, and valtrate led to increased DNA damage of BeWo b30 cells. Results were calculated as fold change compared to the untreated control. Ethyl methanesulfonate (EMS, 3 mM) was used as a positive control (n = 3).

extremely low (not shown); results on the percentage of apoptotic cells concern only the few detectable cells.

Comet assays were performed to determine whether the selected phytochemicals can lead to possible genotoxicity at concentrations up to 10 μM (**Figure 4**). Repetitively, protopine, valerenic acid, and linalool were unremarkable, as predominantly intact nuclear DNA was detected after 3 h of cell treatment; thus none of these substances led to a significant increase in tail DNA. Hyperforin, hypericin, and valtrate showed genotoxicity in this assay, but only when the highest concentration of 10 μM was applied.

The effects of phytochemicals on metabolic parameters, such as glucose and lactate, were examined and expressed as consumption or production, respectively (**Figure 5**). Data were normalized to the protein content. Valerian was the only phytochemical which led to changes in glycolytic metabolism at concentrations of 1, 3, 10, and 30 μM by significantly reducing glucose consumption and lactate production of BeWo b30 cells. None of the remaining phytochemicals (hyperforin, hypericin, protopine, valerenic acid, and linalool) affected the metabolic activity of viable BeWo b30 cells when tested at high concentrations of up to 30 μM. Glucose and lactate concentrations of cell supernatants were not statistically different from those of the untreated control. However, hyperforin led to a significant increase in glucose consumption and a concomitant increase in lactate production at lower concentrations of 1 and 3 μM.

Finally, the impact of the various phytochemicals on the induction of placental cell differentiation was examined, first, by measuring the secretion of the differentiation marker β-hCG (**Figure 6A**), and second, by the opposite approach, namely whether 24 h pre-incubation with the various phytochemicals could inhibit cell differentiation (as induced by FSK; **Figures 6B,C**). In the first measurement, and after the addition of FSK as a positive control, there was a 42-fold increase in β-hCG levels, which is characteristic of BeWo cell differentiation. In contrast, none of the six phytochemicals (hyperforin, hypericin, protopine, valerenic acid, valtrate, and linalool) triggered an increase in β-hCG production in BeWo cells. For the inhibition assay of placental cell differentiation, only non-toxic concentrations of each phytochemical (based on preliminary data, not shown) were chosen (**Figure 6B**). In these assays also, the addition of FSK for 24 h and pre-incubation with cell culture medium for 24 h resulted in 9-fold increased β-hCG levels. Under these conditions, most of the phytochemicals (in different concentrations ranging from 0.03 up to 30 μM) had no statistically significant inhibitory effect on the FSK-induced placenta cell differentiation. However, a 24 h pre-incubation with the highest concentrations of hyperforin (1, 3, and 10 μM) did inhibit the following differentiation of BeWo b30 cells. To confirm this, the assay was repeated on a bigger scale to show the hormone concentration normalized to the amount of protein (μg) (**Figure 6C**). Again, pre-incubation with hypericin

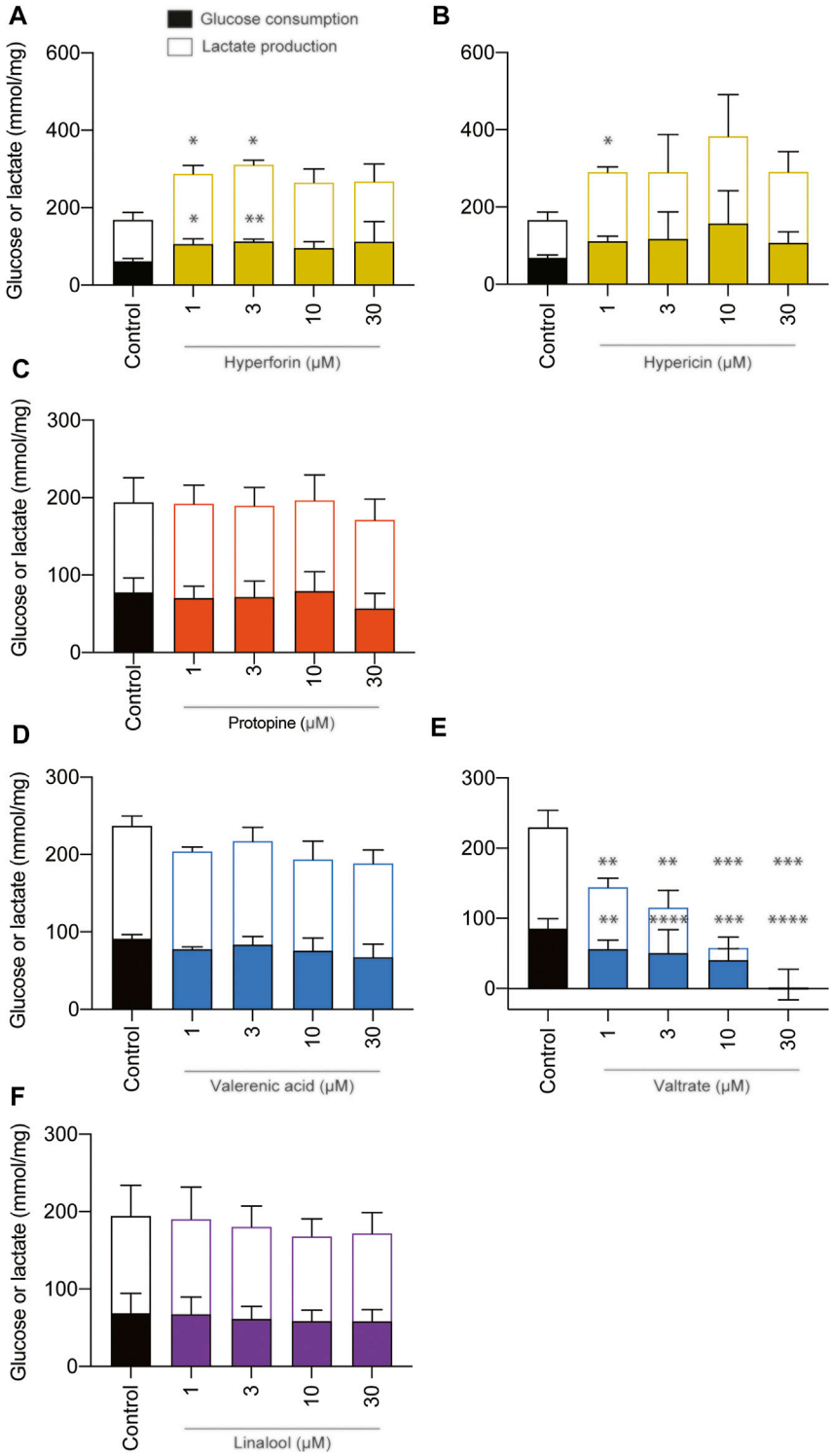

FIGURE 5 | Effects of phytochemicals on glucose consumption and lactate production in undifferentiated BeWo b30 cells after treatment for 48 h. Data were normalized per amount of protein (mg). Control consisted of cell culture media containing 0.2% of DMSO. Data were obtained from three independent experiments (n = 3; in triplicate) and are shown as mean ± SD: *$p < 0.05$. A statistically significant impairment of metabolic activity could not be detected at any of the test concentrations (1, 3, 10, and 30 μM) of the following phytochemicals: protopine **(C)**, valerenic acid **(D)**, and linalool **(F)**. However, valtrate **(E)** decreased the glycolytic metabolism at concentrations of 1, 3, 10, and 30 μM. Phytochemicals of St. John's wort led to increased glucose consumption and concomitant lactate production in the case of hyperforin treatment **(A)** at 1 and 3 μM and increased lactate concentrations in the case of hypericin **(B)** at 1 μM.

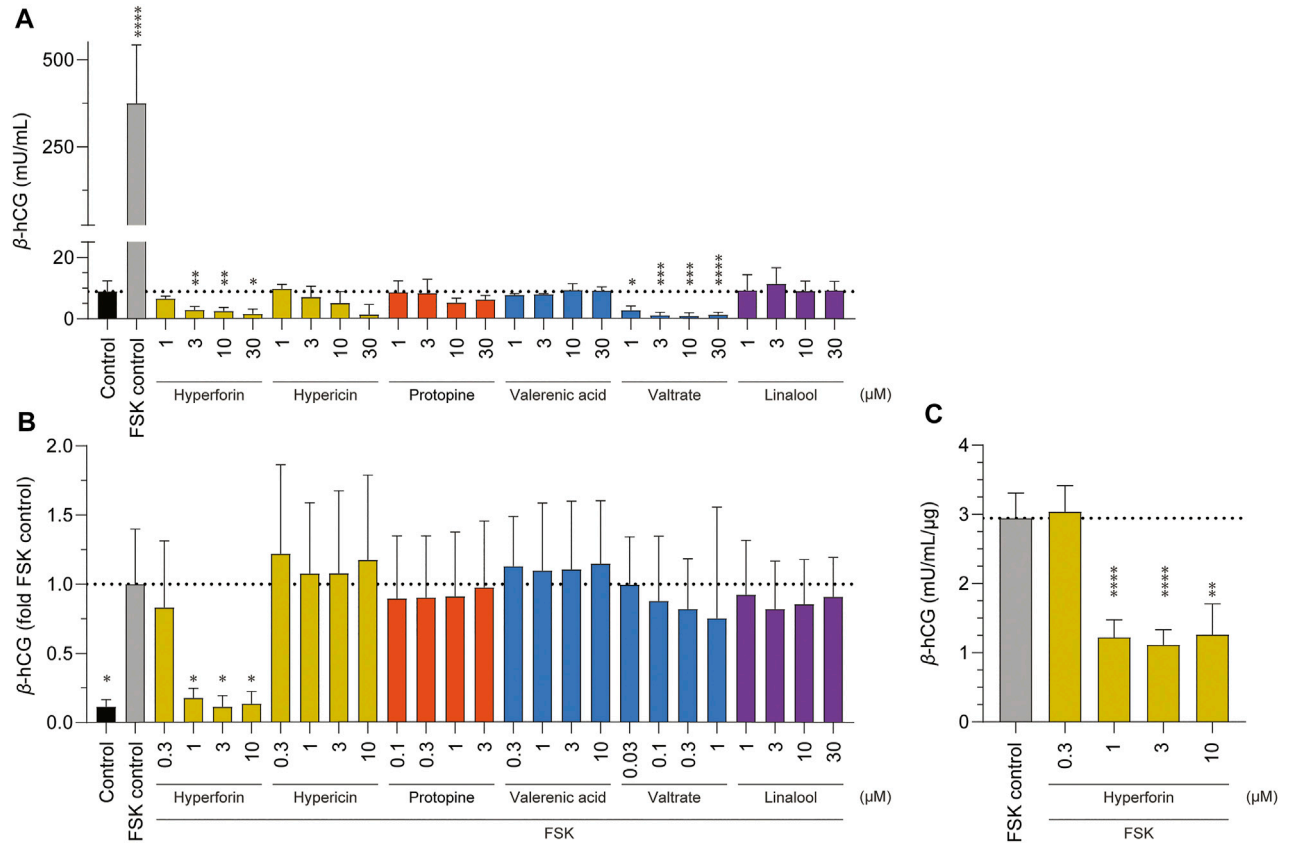

FIGURE 6 | Effects of various phytochemicals on the production of β-hCG in BeWo b30 cells. Control consisted of cell culture media containing 0.2% DMSO; 5 μM forskolin (FSK) was used for the FSK control. Data are presented as mean ± SD of at least three independent experiments (n = 3–4; in triplicate): *p < 0.05. **(A)** Comparison of β-hCG secretion of BeWo b30 cells upon 48-h treatment with increasing concentrations of phytochemicals (1, 3, 10, and 30 μM) vs. FSK control. **(B)** Effects on inhibition of FSK-induced differentiation of BeWo b30 cells. FSK treatment (5 μM) led to increased β-hCG levels in all phytochemicals after an incubation of 48 h, except for hyperforin, where the FSK-induced differentiation was inhibited at concentrations of 1, 3, and 10 μM. Based on preliminary data (not shown), different concentration gradients (ranging from 0.03 up to 30 μM) of phytochemicals were individually determined in advance (before exposure). Cells were pre-treated with the different phytochemicals for 24 h, before the addition of FSK for another 24 h. **(C)** Effects of hyperforin on inhibition of FSK-induced differentiation of BeWo b30 cells normalized per amount of protein (μg). FSK treatment (5 μM) led to decreased β-hCG levels after an hyperforin incubation of 48 h at concentrations of 1, 3, and 10 μM.

(1, 3, and 10 μM) was shown to inhibit FSK-induced differentiation.

4 DISCUSSION

4.1 Main Findings

None of the hydroalcoholic extracts from St. John's wort, California poppy, valerian, and hops, nor lavender essential oil significantly affect the protein expression of BeWo b30 cells after 2 days of incubation at a concentration of 30 μg/ml. When we focused on compounds that might conceivably cause safety issues, no decreased cell viability and induction of apoptosis could be observed in a concentration range between 0.01 and 0.3 μM. However, hypericin (≥1 μM), protopine (30 μM) and valtrate (≥10 μM) led to cytotoxic effects and thus decreased the viability of BeWo b30 cells, while hyperforin (≥3 μM), hypericin (≥1 μM), and valtrate (≥10 μM) induced cell apoptosis. No genotoxic effects were observed for any of the tested phytochemical concentrations of 0.1 and 1 μM. Hyperforin, hypericin, and valtrate were only DNA-damaging at elevated concentrations of 10 μM. A 48-h exposure of valtrate (≥1 μM) resulted in reduced glucose consumption and thus reduced lactate production by placental cells; all incubations with the other phytochemicals at concentrations ranging from 1 to 30 μM resulted in viable cells with normal glycolytic metabolism. None of the tested phytochemicals were able to induce or inhibit BeWo b30 cell differentiation, except hyperforin, which was able to inhibit FSK-induced cell differentiation at concentrations of ≥1 μM. To summarize (**Table 1**), protopine, valerenic acid, and linalool were very inconspicuous in all *in vitro* experiments. Valtrate resulted in cytotoxic, apoptotic, and genotoxic effects (≥10 μM) which were also reflected in reduced metabolic activity (≥1 μM). Hyperforin and hypericin, the two constituents of St. John's

TABLE 1 | Summary of the effects[a] of plant extracts and their constituents on cytotoxicity, genotoxicity, metabolic activity, and their ability to induce or inhibit cell differentiation, in addition to reported plasma concentrations in literature.

Plant/Constituent	Viability	Apoptosis	Genotoxicity	Metabolic Activity		Differentiation		Reported plasma concentrations
				Glucose	Lactate	Induction	Inhibition	
St. John's wort (μg/ml)	↓ (100)	↑ (100)	↔	↔	↔	↔	↔	
Hyperforin (μM)	↔	↑ (3)	↑ (10)	↔	↔	↔	↑ (1)	0.3 (Agrosi et al., 2000)
Hypericin (μM)	↓ (1)	↑ (1)	↑ (10)	↔	↔	↔	↔	0.0088 (Schulz et al., 2005)
California poppy (μg/ml)	↓ (100)	↔	↔	↔	↔	↔	↔	
Protopine (μM)	↓ (30)	↔	↔	↔	↔	↔	↔	n.d
Valerian (μg/ml)	↓ (100)	↑ (100)	↔	↔	↔	↔	↔	
Valerenic acid (μM)	↔	↔	↔	↔	↔	↔	↔	18.2 (Anderson et al., 2005)
Valtrate (μM)	↓ (10)	↑ (10)	↑ (10)	↓ (1)	↓ (1)	↔	↔	n.d
Lavender (μg/ml)	↔	↔	↔	↔	↔	↔	↔	
Linalool (μM)	↔	↔	↔	↔	↔	↔	↔	0.85 (Müller et al., 2015)
Hops (μg/ml)	↓ (100)	↔	↔	↔	↔	↔	↔	

[a]*Only the lowest concentration that did cause a clear-cut effect is shown; n.d. = not determined.*

wort, also showed increased toxicity at concentrations ≥1 μM, with the former inhibiting placental cell differentiation (≥1 μM) as well. To put the potential toxicity of the phytochemicals in perspective, available data on achievable plasma concentrations are also presented in **Table 1**.

4.2 Strengths and Limitations

A major strength of this *in vitro* study is the LC-MS/MS based proteomics approach, which allowed us to study activation or inhibition of pathways important for cell viability, proliferation, and differentiation. Moreover, and as done before with extracts, conceivably critical phytochemicals were studied using a variety of well-established assays with shorter (3 h) and longer (72 h) exposure times, including a wide range of concentrations (from 0.01 up to 30 μM). Because of the lack or limited applicability of pharmacokinetic data, these concentrations were chosen to cover up to a hundred times the maximum achievable plasma concentrations after oral application (compare with (Agrosi et al., 2000)). One limitation of the relevance of our data to everyday practice is that most manufacturers do not provide information on the contents of the individual natural products, which makes it very difficult to discuss physiologically relevant concentration ranges of single phytochemicals. In addition, we here characterized the *in vitro* safety of a few compounds only, which is another limitation since the herbal medicines are multicomponent mixtures; the reader is, however, referred to our previous work (Spiess et al., 2021) for the results obtained with the corresponding extracts in the same functional assays. Finally, it is important to remember that the cell line represents only one layer of the placenta and thus does not fully reflect the biological environment of the placenta barrier and that ADME aspects are not considered in the used *in vitro* models.

4.3 Herbal Medicines and NMD Treatment

In the following, our present observations relevant for a safety assessment of each of the herbal medicines are discussed in the context of available literature.

Incubation with **St. John's wort** extract did not induce relevant changes in protein expression. Out of 4,060 detected proteins, only 3 were up-regulated (RN149, TAP26, and MBOA7) and 1 was down-regulated (PPR21). Functional assays with two biologically highly active phytochemicals revealed that for **hyperforin**, there is no cause for concern about concentrations ≤0.3 μM, as apoptosis (≥3 μM), genotoxicity (10 μM), and inhibition of differentiation (≥1 μM) were increased only at the indicated concentrations. Concentrations of 1 μM of hyperforin are at least 3 times higher than maximum achieved plasma concentrations upon treatment with common preparations. These vary widely depending on daily doses tested, dosing regimen, formulation (soft/hard gelatin capsules), and manufacturer (Agrosi et al., 2000; Schulz et al., 2005; Vitiello et al., 2005). At high concentrations, hyperforin could pose embryotoxic and teratogenic risks since it inhibited the growth and differentiation of embryonic stem cells and induced apoptosis in fibroblasts (Nakamura et al., 2013). The highest plasma concentration recorded in the literature is 168.35 ng/ml of hyperforin after administration of a soft gelatin capsule (300 mg St. John's wort dry extract containing 5% hyperforin and 0.3% hypericin, Hammer Pharma SpA), which converts to approximately 0.3 μM. **Hypericin** showed no toxicity up to a concentration of 0.3 μM, as cytotoxicity, apoptosis, and genotoxicity were increased only above 1, 1, and 10 μM, respectively. Hypericin is less abundant in St. John's wort than hyperforin (Blaschek, 2016), and maximum peak plasma concentrations of 4.43 ng/ml have been reported with multiple dose administration (Schulz et al., 2005), corresponding to approximately 8.8 nM, which is 33 times lower than 0.3 μM. The amount of hypericin as well as hyperforin were recently quantified in different formulations on the Swiss market by HPLC (Schäfer et al., 2019). It was found that the declared and the actually quantified contents differed considerably in some cases. In addition, all formulations contained hypericin, but hyperforin

was not detected in two products (Vogel HyperiMed® and Vogel Hyperiforce®) and its amount in other products was very low (Rebalance® and Remotiv®). Since we detected an inhibition of differentiation starting at 1 μM of hyperforin and any alteration in the physiological development of the placenta might be critical from a safety point of view, in our opinion formulations with a low hyperforin content could be preferred in pregnancy. In our previous study on whole extracts, St. John's wort showed no significant effects up to a concentration of 30 μg/ml extract for all types of *in vitro* experiments, namely cell viability, apoptosis, genotoxicity, metabolic activity, and inhibition/induction of placental cell differentiation (Spiess et al., 2021). In general, there is a lack of adequate clinical studies on the genotoxicity of St. John's wort, as well as tests on reproductive toxicity, and fertility (Greeson et al., 2001; Avila et al., 2018). Well known issues such as phototoxicity (Onoue et al., 2011) and interactions with other medications (Nicolussi et al., 2020) must be taken care of also during administration in pregnancy. Moreover, a few studies which were either prospective (Kolding et al., 2015) or based on data analysis of a national birth cohort (Moretti et al., 2009) or on claim data (Schäfer et al., 2021) seem to suggest an increased risk of fetal malformation in pregnant women exposed to St. John's wort preparations. While the results are striking, they should be interpreted with caution.

In the case of **California poppy**, there were no relevant changes in protein expression when BeWo b30 placental cells were incubated with the extract for 2 days; only one protein (ANR35) was significantly upregulated (1/3,353). California poppy, and several other medicinal plants of the Papaveraceae family (e.g., Papaver, Chelidonium, and Argemone, etc.) as well as of other plant families (e.g., Berberidaceae, Fumariaceae, and Ranunculaceae, etc.) (Guinaudeau and Shamma, 1982), contains **protopine**, and this phytochemical was also investigated. We found a minimal cytotoxic potential, as only the viability assay showed slight significance at the highest concentration of 30 μM. To the best of our knowledge, there are no reports of pharmacokinetic studies of protopine in the literature, which makes it difficult to compare the test concentrations we used with physiologically relevant plasma concentrations in humans. Especially ADME studies are needed to predict any *in vivo* effects of protopine. How much protopine is present in commercially available preparations from California poppy is also unknown and requires further clarification. Our previous (Spiess et al., 2021) and current *in vitro* contributions to the study of California Poppy (and protopine) would be in line with a good safety profile.

The proteomics approach is also indicative of good safety for **valerian**. Incubation with the corresponding extract led to no relevant changes in the proteome. In fact, only 2 proteins (COPRS, AN32B) out of 3,423 were significantly upregulated, and 1 protein (FLOT2) was downregulated. In our various experimental set-ups, two biologically active phytochemicals were included. **Valerenic acid** showed neither toxicological effects, nor significant effects on metabolic properties (glucose consumption, lactate production) nor differentiation of BeWo b30 cells in a concentration range between 0.01–30 μM. **Valtrate**, however, showed significant toxic effects, as cytotoxicity, apoptosis, and genotoxicity were increased under the influence of ≥10 μM. In addition, valtrate was also the only ingredient that had significant effects on the metabolic properties of BeWo b30 cells at levels as low as 1 μM. From a translational perspective, these effects must be interpreted with caution, as valepotriates (like valtrate) are very unstable and are easily degraded by exposure to heat, acids or bases (Blaschek, 2016). Also, in the extract used in our previous (Spiess et al., 2021) and present study no valtrate was present. To our knowledge, no data on plasma concentrations of valtrate exist. For valerenic acid, a maximum serum level of 2.8 μg/ml (i.e. 18.2 μM) was reported in one subject after a single administration of 600 mg valerian (Sedonium™, Lichtwer Pharma) via indwelling catheter in the arm vein (Anderson et al., 2005). Upon oral application, lower plasma concentrations are to be expected. As a reminder, we included concentrations of valerenic acid of up to 30 μM in most assays of our study. Since even at these concentrations no effect was observed and, in addition, valerian extract (≥30 μg/ml) had no negative effect on BeWo b30 cells from a toxicological and metabolic point of view, as well as on placental cell differentiation, our work is in line with good safety for valerian in pregnancy. Studies with animals also found that orally administered valerian extract had no adverse effects on fertility or fetal development (Yao et al., 2007).

Finally, **lavender essential oil** did not affect protein expression in any relevant way. Of the 3,999 proteins identified, only 24 were significantly up- (3 proteins) or downregulated (21 proteins). Importantly, no explicit pathway was overly involved. Lavender oil was also highly inconspicuous in all functional assays from our previous study (Spiess et al., 2021). The same assays were now performed with **linalool**, one of the two major components of the essential oil. The results revealed no impairment of BeWo cells at concentrations up to 30 μM initial concentration. Additional experiments showed that some linalool volatizes during the incubations, therefore with time this initial concentration might have been reduced by up to 64% (30 μM would then be reduced to approximately 10 μM). Of particular importance is the comparison with maximum determined plasma concentrations of linalool (Lasea®, Dr. Willmar Schwabe GmbH and Co KG), where peak values of 22 ng/ml (≙ 0.14 μM) and 131 ng/ml (≙ 0.85 μM) were reached after single administration of 80 mg (therapeutic dose) or multiple administrations (14-days application) of 160 mg, respectively (Müller et al., 2015). These concentrations are therefore markedly lower than those used in our study, even when accounting for volatilization. Plasma concentrations upon liniments or embrocations with lavender oil are not known. Regardless of pregnancy, attention should be drawn to the common adverse reactions (<1/10, ≥1/100) such as gastrointestinal disturbances (gastroesophageal reflux, nausea) and allergic skin reactions (urticaria, pruritus, exanthema) (Medicinal product information search platform (AIPS), 2022).

The **hops** extract was very inconspicuous in our proteomics studies, as out of a total of 3,387 proteins only one (NAGAB) was significantly upregulated. Various *in vitro* toxicity assays showed no negative effects either up to 30 μg/ml (cytotoxicity) or even 100 μg/ml (apoptosis, genotoxicity, metabolic activity, and influence on differentiation) (Spiess et al., 2021). To our knowledge there are no concerns about a specific phytochemical from hops. To date, there are no hop cone mono-preparations available in Switzerland. Their dry extracts are often combined with well-established medicinal plants such as valerian root or passionflower herb, as their effects complement each other well (Medicinal product information search platform (AIPS), 2022). Our data imply no significant harm from a toxicological perspective.

5 FINAL STATEMENT

In conclusion, the herbal extracts and some of their constituents of St. John's wort (hyperforin, hypericin), California poppy (protopine), valerian (valerenic acid, valtrate), lavender (linalool), and hops showed no toxicological abnormalities in a relevant (low) concentration range, suggesting that low doses of these herbal medicines are likely to be safe during pregnancy. Since hyperforin was able to inhibit placental cell differentiation of cytotrophoblasts into syncytiotrophoblasts (≥1 μM), St. John's wort formulations with a low hyperforin content should preferably be consumed during pregnancy. Further experimental work should focus on potential fetal exposure, evaluating the transplacental transport of hyperforin—and other compounds that might affect relevant cellular processes and/or cause genotoxic effects. Finally and in view of the urgent need for herbal medicines as treatment options for NMDs in pregnancy, more prospective clinical studies should be conducted to evaluate both efficacy and safety of the most promising herbal medicines.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/pride/archive/, PXD031765.

AUTHOR CONTRIBUTIONS

APS-W and CG designed the study. DS and MW planned and performed the experiments and analyzed the data. DS focused on the proteomics analysis, metabolic properties, and differentiation of placenta cells, and wrote the first version of the manuscript under the supervision of APS-W. MW conducted experiments on the viability, apoptosis, and genotoxicity of placenta cells. ADB performed parts of the experiments and especially supported with the sample preparation for protein analysis. All authors were engaged in revising the manuscript and agreed with the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.882997/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor MH declared a past co-authorship with the author CG.

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Chapter 3 – Supporting Information

Advanced *in vitro* safety assessment of herbal medicines for the treatment of non-psychotic mental disorders in pregnancy

Deborah Spiess, Moritz Winker, Alexandra Dolder Behna, Carsten Gründemann, Ana Paula Simões-Wüst

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Table 1S. Identified proteins which were significantly influenced (up- or downregulated) after incubations with plant extracts for a period of 48 h in comparison with vehicle only (DMSO treatment).

Plant extract	Gene	Protein	Function [1, 2]	log_2FC	p-value	Adjusted p-value
St. John's wort	RN149	E3 ubiquitin-protein ligase	Enables E3 ubiquitin-protein ligase activity	2.60	8.65E-06	0.018
	TAP26	Thyroid transcription factor 1-associated protein 26	Component of the transcription complexes of the pulmonary surfactant-associated protein-B (SFTPB) and -C (SFTPC) which enhances the homeobox protein Nkx-2.1-activated SFTPB and SFTPC promoter activities	1.80	7.52E-05	0.076
	MBOA7	Lysophospholipid acyltransferase 7	Contributes to the regulation of free arachidonic acid in the cell through the remodeling of phospholipids	1.27	7.29E-05	0.076
	PPR21	Protein phosphatase 1 regulatory subunit 21	Regulator of protein phosphatase 1	-2.73	9.15E-05	0.076
California poppy	ANR35	Ankyrin repeat domain-containing protein 35	Unknown	4.68	1.92E-05	0.066
Valerian	COPRS	Coordinator of PRMT5 and differentiation stimulator	Histone-binding protein which plays a role in muscle cell differentiation	2.92	1.02E-04	0.119
	AN32B	Acidic leucine-rich nuclear phosphoprotein 32 family member B	Multifunctional protein which is involved in the regulation of many processes e.g. cell proliferation, apoptosis, cell cycle progression or transcription	2.67	3.98E-06	0.014
	FLOT2	Flotillin-2	Functionally participating in formation of caveolae or caveolae-like vesicles	-1.38	3.31E-05	0.058
Lavender	CALB2	Calretinin	Calcium-binding protein which is abundant in auditory neurons	1.82	1.50E-04	0.111
	SPTB1	Spectrin beta chain, erythrocytic	Major constituent of the cytoskeletal network underlying the erythrocyte plasma membrane	1.36	7.69E-04	0.142
	GEMI	Geminin	Inhibits DNA replication	1.13	1.08E-03	0.166
	BGLR	Beta-glucuronidase	Plays an important role in the degradation of dermatan and keratan sulfates	-1.01	4.24E-04	0.123

UBQL2	Ubiquilin-2	Plays an important role in the regulation of different protein degradation mechanisms and pathways including ubiquitin-proteasome system (UPS), autophagy and the endoplasmic reticulum-associated protein degradation (ERAD) pathway.	-1.04	5.88E-05	0.080
RIOX2	Ribosomal oxygenase 2	Leads to an increase in ribosomal RNA expression; may play an important role in cell growth and survival and may be involved in ribosome biogenesis	-1.06	3.70E-04	0.123
STT3B	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit STT3B	Plays a role in ER-associated degradation (ERAD) pathway that mediates ubiquitin-dependent degradation of misfolded endoplasmic reticulum proteins; required for efficient post-translational glycosylation	-1.07	1.25E-03	0.181
STT3A	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit STT3A	Similar catalytic subunit with an overlapping function as STT3B	-1.09	4.21E-04	0.123
SET	Protein SET	Multitasking protein, involved in apoptosis, transcription, nucleosome assembly and histone chaperoning	-1.17	2.74E-04	0.123
PEBB	Core-binding factor subunit beta	Master-regulates a host of genes specific to hematopoiesis and osteogenesis	-1.22	5.46E-04	0.142
SC11A	Signal peptidase complex catalytic subunit SEC11A	Component of the microsomal signal peptidase complex which removes signal peptides from nascent proteins as they are translocated into the lumen of the endoplasmic reticulum	-1.23	8.02E-04	0.142
TTC9C	Tetratricopeptide repeat protein 9C	Protein coding gene; important paralog of the TTC9 gene which has been shown to be hormonally regulated in breast cancer cells and may play a role in cancer cell invasion and metastasis	-1.24	2.23E-04	0.115
TBA1B	Tubulin alpha-1B chain	Major constituent of microtubules	-1.25	7.26E-04	0.142
CCS	Copper chaperone for superoxide dismutase	Delivers copper to copper zinc superoxide dismutase	-1.31	2.25E-04	0.115
CACO2	Calcium-binding and coiled-coil domain-containing protein 2	Required for autophagy-mediated intracellular bacteria degradation	-1.32	3.43E-04	0.123
GALK1	Galactokinase	Major enzyme for galactose metabolism	-1.50	1.08E-04	0.110
TLE1	Transducin-like enhancer protein 1	Transcriptional corepressor that binds to a number of transcription factors	-1.71	1.48E-05	0.030

	TYSY	Thymidylate synthase	Contributes to the de novo mitochondrial thymidylate biosynthesis pathway	-1.72	5.96E-04	0.142
	COQ9	Ubiquinone biosynthesis protein COQ9, mitochondrial	Lipid-binding protein involved in the biosynthesis of coenzyme Q, also named ubiquinone, an essential lipid-soluble electron transporter for aerobic cellular respiration	-1.81	5.67E-04	0.142
	RL36	60S ribosomal protein L36	Component of the large ribosomal subunit	-1.94	1.64E-04	0.111
	TF2AA	Transcription initiation factor IIA subunit 1	Component of the transcription machinery of RNA polymerase II and plays an important role in transcriptional activation	-2.15	4.00E-04	0.123
	URM1	Ubiquitin-related modifier 1	Involved in a specific pathway, which is part of tRNA modification	-2.31	4.15E-07	0.002
	S61A1	Protein transport protein Sec61 subunit alpha isoform 1	Plays a crucial role in the insertion of secretory and membrane polypeptides into the endoplasmic reticulum	-2.45	7.85E-04	0.142
	EIF3D	Eukaryotic translation initiation factor 3 subunit D	mRNA cap-binding component of the eukaryotic translation initiation factor 3 (eIF-3) complex, a complex required for several steps in the initiation of protein synthesis of a specialized repertoire of mRNAs	-2.86	1.08E-03	0.166
Hops	NAGAB	Alpha-N-acetylgalactosaminidase	Required for the breakdown of glycolipids	2.30	3.59E-06	0.013

Table 2S. Label-free quantification of proteins which were only identified in either the treated (St. John's wort extract) or untreated (0.06% DMSO) sample.

	Gene	Protein	Function [1, 2]
Treated (St. John's wort)	CDIPT	CDP-diacylglycerol-inositol 3-phosphatidyltransferase	Catalyzes the biosynthesis of phosphatidylinositol (PtdIns) as well as PtdIns:inositol exchange reaction
	PPAL	Lysosomal acid phosphatase	Hydrolyzes orthophosphoric monoesters to alcohol and phosphate
	AT12A	Potassium-transporting ATPase alpha chain 2	Catalytic subunit of a H^+/K^+ and/or Na^+/K^+ -ATPase pump which transports K^+ ions (into the cell) in exchange for Na^+ and/or H^+ ions (to the extracellular compartment) across the apical membrane of epithelial cells
Untreated (0.06% DMSO)	TGT	Queuine tRNA-ribosyltransferase catalytic subunit 1	Catalyzes the base-exchange of a guanine (G) residue with queuine (Q) at position 34 (anticodon wobble position) in tRNAs with GU_N anticodons (tRNA-Asp, -Asn, -His and -Tyr), resulting in the hypermodified nucleoside queuosine
	5NT3A	Cytosolic 5'-nucleotidase 3A	Specific activity towards cytidine monophosphate (CMP) and 7-methylguanosine monophosphate (m_7GMP); CMP seems to be the preferred substrate

Table 3S. Label-free quantification of proteins which were only identified in either the treated (valerian extract) or untreated (0.06% DMSO) sample.

	Gene	Protein	Function [1, 2]
Treated (valerian)	WIPF1	WAS/WASL-interacting protein family member 1	Plays a role in the reorganization of the actin cytoskeleton; contributes with NCK1 and GRB2 in the recruitment and activation of WASL; may participate in regulating the subcellular localization of WASL, resulting in the disassembly of stress fibers in favor of filopodia formation; plays a role in the formation of cell ruffles
	MBP	Myelin basic protein	Is, with PLP, the most abundant protein component of the myelin membrane in the CNS; formation and stabilization of this compact multilayer arrangement of bilayers

Untreated (0.06% DMSO)	MYL3	Myosin light chain 3	Regulatory light chain of myosin; does not bind calcium
	1B41	HLA class I histocompatibility antigen, B-41 alpha chain	Antigen-presenting major histocompatibility complex class I (MHCI) molecule; in complex with B2M/beta 2 microglobulin displays primarily viral and tumor-derived peptides on antigen-presenting cells for recognition by alpha-beta T cell receptor (TCR) on HLA-B-restricted CD8-positive T cells, guiding antigen-specific T cell immune response to eliminate infected or transformed cells
	CAVN1	Caveolae-associated protein 1	Plays an important role in caveolae formation and organization; essential for the formation of caveolae in all tissues

Table 4S. Label-free quantification of proteins which were only identified in either the treated (hops extract) or untreated (0.06% DMSO) sample.

	Gene	Protein	Function [1, 2]
Treated (hops)	NGRN	Neugrin	Plays an essential role in mitochondrial ribosome biogenesis
	ANKZ1	Ankyrin repeat and zinc finger domain-containing protein 1	Plays a role in the cellular response to hydrogen peroxide and in the maintenance of mitochondrial integrity under conditions of cellular stress; involved in the endoplasmic reticulum (ER)-associated degradation (ERAD) pathway
	DPY30	Protein dpy-30 homolog	Involved in the methylation of histone H3 at 'Lys-4', particularly trimethylation, which represents a specific tag for epigenetic transcriptional activation; may also play an indirect or direct role in endosomal transport
	CLAP1	CLIP-associating protein 1	Microtubule plus-end tracking protein that promotes the stabilization of dynamic microtubules; involved in the nucleation of noncentrosomal microtubules originating from the trans-Golgi network (TGN)
	ITA3	Integrin alpha-3	Integrin alpha-3/beta-1 is a receptor for fibronectin, laminin, collagen, epiligrin, thrombospondin and CSPG4
	SP100	Nuclear autoantigen Sp-100	Together with PML, this tumor suppressor is a major constituent of the PML bodies, a subnuclear organelle involved in a large number of physiological processes including cell growth, differentiation and apoptosis; may play a role in angiogenesis, controlling endothelial cell motility and invasion

	LRCH4	Leucine-rich repeat and calponin homology domain-containing protein 4	This gene encodes a protein that contains leucine-rich repeats (LRR) at its amino terminus and that is known to be involved in ligand binding; carboxyl terminus may act as a membrane anchor
	SET1A	Histone-lysine N-methyltransferase SETD1A	Methylates 'Lys-4' of histone H3, when part of the SET1 histone methyltransferase (HMT) complex, but not if the neighboring 'Lys-9' residue is already methylated; H3 'Lys-4' methylation represents a specific tag for epigenetic transcriptional activation; may play a role in synaptic function and the development of neurons
Untreated (0.06% DMSO)	HMCES	Abasic site processing protein HMCES	Acts as an enzyme that recognizes and binds abasic sites in ssDNA at replication forks and chemically modifies the lesion by forming a covalent cross-link with DNA; promotes error-free repair of abasic sites by acting as a 'suicide' enzyme that is degraded, thereby protecting abasic sites from translesion synthesis (TLS) polymerases and endonucleases that are error-prone and would generate mutations and double-strand breaks; acts as a protease: mediates autocatalytic processing of its N-terminal methionine in order to expose the catalytic cysteine
	GPX4	Phospholipid hydroperoxide glutathione peroxidase	Essential antioxidant peroxidase that directly reduces phospholipid hydroperoxide even if they are incorporated in membranes and lipoproteins; plays a key role in protecting cells from oxidative damage by preventing membrane lipid peroxidation
	PRIO	Major prion protein	Unclear primary physiological function; may play a role in neuronal development and synaptic plasticity; may play a role in iron uptake and iron homeostasis

Table 5S. Label-free quantification of proteins which were only identified in either the treated (California Poppy extract) or untreated (0.06% DMSO) sample.

	Gene	Protein	Function [1, 2]
Treated (California poppy)	NOL3	Nucleolar protein 3	Isoform 1: May be involved in RNA splicing Isoform 2: Functions as an apoptosis repressor that blocks multiple modes of cell death; inhibits extrinsic apoptotic pathways; inhibits intrinsic apoptotic pathway in response to a wide range of stresses, through its interaction with BAX resulting in BAX inactivation, preventing mitochondrial dysfunction and release of pro-apoptotic factors; Inhibits calcium-mediated cell death by functioning as a cytosolic calcium buffer, dissociating its interaction with CASP8 and maintaining calcium homeostasis; negatively regulates hypoxia-induced apoptosis in part by inhibiting the release of cytochrome c from mitochondria in a caspase-independent manner; inhibits TNF-induced necrosis by preventing TNF-signaling pathway through TNFRSF1A interaction abrogating the recruitment of RIPK1 to complex I; promotes vascular remodeling through inhibition of apoptosis and stimulation of proliferation, in response to hypoxia
Untreated (0.06% DMSO)	NUDT3	Diphosphoinositol polyphosphate phosphohydrolase 1	May play a role in signal transduction; acts as a negative regulator of the ERK1/2 pathway
	GSTM1	Glutathione S-transferase Mu 1	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles; involved in the formation of glutathione conjugates of prostaglandin A2 (PGA2) and prostaglandin J2 (PGJ2)
	PURB	Transcriptional activator protein Pur-beta	Capacity to bind repeated elements in single-stranded DNA; plays a role in the control of vascular smooth muscle (VSM) alpha-actin gene transcription as repressor in myoblasts and fibroblasts; participates in transcriptional and translational regulation of alpha-MHC expression in cardiac myocytes by binding to the purine-rich negative regulatory (PNR) element

Untreated (0.06% DMSO)	YJU2	Splicing factor YJU2	Part of the spliceosome which catalyzes two sequential transesterification reactions, first the excision of the non-coding intron from pre-mRNA and then the ligation of the coding exons to form the mature mRNA; may protect cells from TP53-dependent apoptosis upon dsDNA break damage through association with PRP19-CD5L complex
	SPCS1	Signal peptidase complex subunit 1	Component of the microsomal signal peptidase complex which removes signal peptides from nascent proteins as they are translocated into the lumen of the endoplasmic reticulum; plays a key role in the post-translational processing of flaviviral structural proteins prM, E, and NS1

Table 6S. Label-free quantification of proteins which were only identified in either the treated (lavender extract) or untreated (0.06% DMSO) sample.

	Gene	Protein	Function [1, 2]
Treated (lavender)	AAAS	Aladin	Plays a role in the normal development of the peripheral and central nervous system
	PNKP	Bifunctional polynucleotide phosphatase/kinase	Plays a key role in the repair of DNA damage, functioning as part of both the non-homologous end-joining (NHEJ) and base excision repair (BER) pathways; through its two catalytic activities, PNK ensures that DNA termini are compatible with extension and ligation by either removing 3'-phosphates from, or by phosphorylating 5'-hydroxyl groups on, the ribose sugar of the DNA backbone
	REX1B	Required for excision 1-B domain-containing protein	Uncharacterized protein
	FHL3	Four and a half LIM domains protein 3	Recruited by SOX15 to FOXK1 promoters where it acts as a transcriptional coactivator of FOXK1
	CO4A5	Collagen alpha-5(IV) chain	Type IV collagen is the major structural component of glomerular basement membranes (GBM), forming a 'chicken-wire' meshwork together with laminins, proteoglycans and entactin/nidogen

	SEM7A	Semaphorin-7A	Plays an important role in integrin-mediated signaling and functions both in regulating cell migration and immune responses. Promotes formation of focal adhesion complexes, activation of the protein kinase PTK2/FAK1 and subsequent phosphorylation of MAPK1 and MAPK3; promotes production of proinflammatory cytokines by monocytes and macrophages; plays an important role in modulating inflammation and T-cell-mediated immune responses; promotes axon growth in the embryonic olfactory bulb
	PJA2	E3 ubiquitin-protein ligase Praja-2	Responsible for ubiquitination of cAMP-dependent protein kinase type I and type II-alpha/beta regulatory subunits and for targeting them for proteasomal degradation; essential for PKA-mediated long-term memory processes
	MGST3	Microsomal glutathione S-transferase 3	Catalyzes the oxydation of hydroxy-fatty acids; catalyzes the conjugation of a reduced glutathione to leukotriene A4 in vitro; may participate in the lipid metabolism
	FBLL1	rRNA/tRNA 2'-O-methyltransferase fibrillarin-like protein 1	S-adenosyl-L-methionine-dependent methyltransferase that has the ability to methylate both RNAs and proteins; involved in pre-rRNA processing by catalyzing the site-specific 2'-hydroxyl methylation of ribose moieties in pre-ribosomal RNA; also acts as a protein methyltransferase by mediating methylation of glutamine residues
Untreated (0.06% DMSO)	DC1I1	Cytoplasmic dynein 1 intermediate chain 1	Acts as one of several non-catalytic accessory components of the cytoplasmic dynein 1 complex that are thought to be involved in linking dynein to cargos and to adapter proteins that regulate dynein function; cytoplasmic dynein 1 acts as a motor for the intracellular retrograde motility of vesicles and organelles along microtubules; may play a role in mediating the interaction of cytoplasmic dynein with membranous organelles and kinetochores
	RASF8	Ras association domain-containing protein 8	Essential for maintaining adherens junction function in epithelial cells and has a role in epithelial cell migration.

Table 7S. Relative concentrations of linalool present in cell culture medium at different positions of a 96-well or 24-well plate after 3 h or 24 h, respectively.

Plate	Time (h)	Position	Well	Area (GC)	Linalool (%)*	Mean (%)
96-well	0	Stock	-	21395		100
				22767		
				23020		
	3	Center	D5	18468	86	85
				18293	80	
				20494	89	
		Border	A5	12865	60	60
				14684	64	
				12946	56	
24-well	0	Stock	-	410938		100
				381251		
				369752		
	48	Center	B4	165974	40	44
				172929	45	
				167115	45	
			C4	153577	37	41
				159509	42	
				163896	44	
		Border	A1	142483	35	36
				117050	31	
				152353	41	

* Calculated as (area t_{24})/(area stock t_0)*100.

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Chapter 4

Chapter 4

Placental passage of protopine in an *ex vivo* human perfusion system

Deborah Spiess[*], Vanessa Fabienne Abegg[*], Antoine Chauveau, Andrea Treyer, Michael Reinehr, Mouhssin Oufir, Elisa Duong, Olivier Potterat, Matthias Hamburger, Ana Paula Simões-Wüst

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The placental passage of protopine was investigated with a human *ex vivo* placental perfusion model. The model was first validated with diazepam and citalopram, 2 compounds known to cross the placental barrier, and antipyrine as a positive control. All compounds were quantified by partially validated U(H)PLC-MS/MS bioanalytical methods. Protopine was transferred from the maternal to the fetal circuit, with a steady-state reached after 90 min. The study compound did not affect placental viability or functionality, as glucose consumption, lactate production, and beta-human chorionic gonadotropin, and leptin release remained constant. Histopathological evaluation of all placental specimens showed unremarkable, age-appropriate parenchymal maturation with no pathologic findings.

Contributions of Deborah Spiess to this publication: performing placental perfusion experiments, analysis and interpretation of data, data visualization, writing the first manuscript draft, and manuscript revision

[*] *These authors contributed equally to the work and should be considered as joint first authors*

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Placental Passage of Protopine in an *Ex Vivo* Human Perfusion System

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Authors
Deborah Spiess[1,2*], Vanessa Fabienne Abegg[2*], Antoine Chauveau[2], Andrea Treyer[2], Michael Reinehr[3], Mouhssin Oufir[2**], Elisa Duong[1,2], Olivier Potterat[2], Matthias Hamburger[2], Ana Paula Simões-Wüst[1]

Affiliations
1 Department of Obstetrics, University Hospital Zurich, University of Zurich, Zurich, Switzerland
2 Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland
3 Department of Pathology and Molecular Pathology, University Hospital Zurich, Zurich, Switzerland

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Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

Correspondence
Prof Dr Matthias Hamburger
Department of Pharmaceutical Sciences, Division of Pharmaceutical Biology, University of Basel
Klingelbergstrasse 50, 4056 Basel, Switzerland
Phone: + 41 6 12 07 14 25, Fax: + 41 6 12 07 14 74
matthias.hamburger@unibas.ch

Correspondence
PD Dr Ana Paula Simões-Wüst
Department of Obstetrics, University Hospital of Zurich
Schmelzbergstrasse 12/PF 125, Path G51a, 8091 Zurich, Switzerland
Phone: + 41 4 42 55 51 31, Fax: + 41 4 42 55 44 30
AnaPaula.Simoes-Wuest@usz.ch

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ABSTRACT

The placental passage of protopine was investigated with a human *ex vivo* placental perfusion model. The model was first validated with diazepam and citalopram, 2 compounds known to cross the placental barrier, and antipyrine as a positive control. All compounds were quantified by partially validated U(H)PLC-MS/MS bioanalytical methods. Protopine was transferred from the maternal to the fetal circuit, with a steady-state reached after 90 min. The study compound did not affect placental viability or functionality, as glucose consumption, lactate production, and beta-human chorionic gonadotropin, and leptin release remained constant. Histopathological evaluation of all placental specimens showed unremarkable, age-appropriate parenchymal maturation with no pathologic findings.

* These authors contributed equally to the work.

** Present address: Oncodesign SA, Villebon-sur-Yvette, France

ABBREVIATIONS

96-DWP	96-deepwell plate
Cal	calibration sample
CHMP	Committee on Herbal Medicinal Products
EMA	European Medicines Agency
ESI	electrospray ionization
FITC	fluorescein isothiocyanate
FM	fetal-maternal
FM ratio	fetal-maternal concentration ratio
IS	internal standard
LLOQ	lower limit of quantification
MeCN	acetonitrile
NMD	non-psychotic mental disorder
PM	perfusion medium
QC	quality control
QCH	quality controls at high levels
QCL	quality controls at low levels
QCM	quality controls at medium levels
SS	stock solution
ULOQ	upper limit of quantification
WS	working solution
***β*-hCG**	beta-human chorionic gonadotropin

Introduction

During pregnancy, a large number of women need medical care. Pharmacotherapy in pregnant women is challenging, given that adverse effects on the embryo/fetus have to be considered [1]. The situation is exacerbated by the fact that pregnant women are, in most cases, actively excluded from clinical drug development trials. This severely reduces the number of medications labeled for use during pregnancy [2, 3]. As a consequence, clinicians often make use of the so-called off-label prescribing (i.e., they advise the use of medications in a way that diverges from the approved product information [e.g., indication, application, dosage, patient categories]) [4]. Probably for all these reasons, expectant mothers often perceive synthetic medications as potentially dangerous. They try to reduce their consumption [5, 6] and seek supposedly safe alternatives, such as phytomedicines. In a multinational study, an average of 28.9% of pregnant women reported using herbal medicines during pregnancy, with an even higher proportion of 40.6% in Switzerland [7].

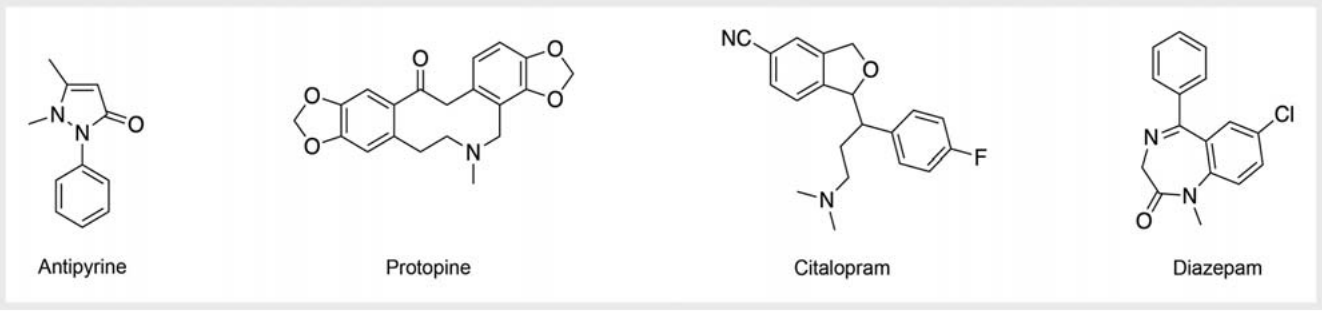
Some of the phytomedicines are used in the treatment of non-psychotic mental disorders (NMDs) in pregnancy, such as sleep disorders, restlessness, anxiety, and mild depression. A recent prevalence estimate in Switzerland reported that 16.7% of perinatal women used mental healthcare [8].

The perception of phytomedicines as safe in pregnancy [9] contradicts that studies on their safety in pregnancy are essentially lacking. For example, how much phytochemicals can pass across the placental barrier to reach the fetus is unknown. We are currently investigating the transplacental transfer of selected compounds from medicinal plants used to treat mild NMDs in pregnancy to shed some light on the matter.

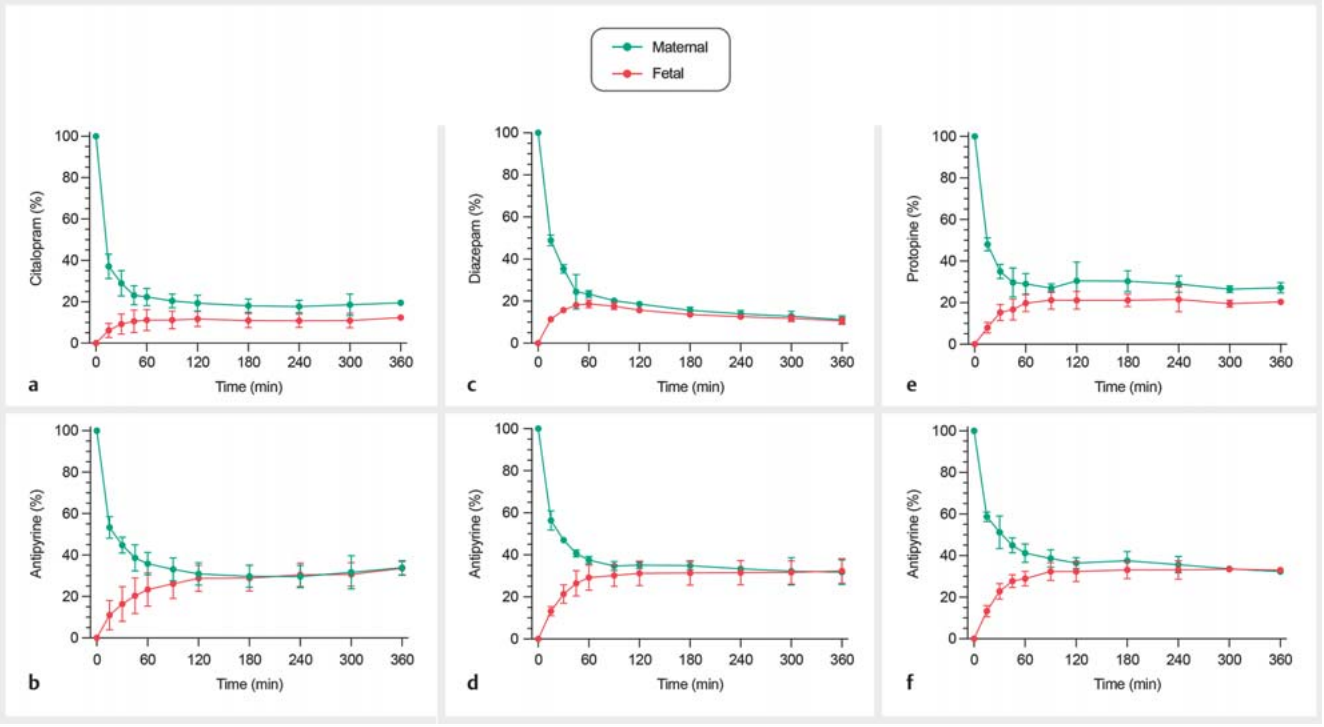
California poppy (*Eschscholzia californica* Cham., Papaveraceae) has a long tradition among indigenous people in the USA [10]. The CHMP has classified it for traditional use as a sleeping aid and for the relief of mild symptoms of mental stress [11]. Various *Eschscholzia* products are on the market, ranging from approved phytomedicines to food supplements available via the internet. They contain either powdered herbal drug or extract as the active ingredient, sometimes combined with other herbs. Very few products have been standardized for their content in total alkaloids [12–14]. California poppy contains 0.5% to 1.2% of total alkaloids, with protopine, a phytochemical that is also present in several other herbal medicines [15], being one of the major compounds [16–18]. Extracts of California poppy have shown sedative and anxiolytic effects *in vivo* [10], and these properties have been attributed to the isoquinoline alkaloids [19]. Several *in vitro* studies suggest that protopine is a CNS-active compound. Protopine was found to bind with $GABA_A$ receptors in rat synaptic membrane preparations [20, 21]. Protopine was also shown to be a ligand at $5\text{-}HT_{1A}$ receptors expressed in human CHO cell membranes [22]. The alkaloid is also an inhibitor of serotonin and noradrenaline transporters expressed in murine S6 and N1 cells, respectively [23]. The CHMP does not recommend using California poppy during pregnancy due to a lack of sufficient safety data [11].

A broad range of *in vitro* and *in vivo* models have been used to assess fetal exposure to exogenous compounds. Chronically cannulated sheep have been used extensively, and *in situ* placental perfusion techniques in rodents (guinea pigs, rabbits) have been established [24]. *Ex vivo* perfusion models with rats [25] and mice [26] have been used for an early screening of substance transfer across the placental barrier. However, with all animal models, the extrapolation of results to humans is limited due to functionally and anatomically large interspecies differences [24, 27, 28]. *In vitro* models utilizing well-established human placental cell lines (e.g., BeWo, Jar, JEG-3 cells) or human placental primary cells (villous trophoblasts) and explant tissue have been employed. These latter models enable the study of various factors affecting the transplacental transport, such as uptake, efflux, and metabolism. BeWo b30 cells (a clone of BeWo cells) form confluent monolayers on the semi-permeable membrane of Transwell inserts can be used as an *in vitro* model for the placental barrier. However, all cell-based placental models lack the cellular organization, compartmentalization and 3-dimensional structure of intact, physiologically active placentae [29]. The current gold standard among the placental transfer models is the *ex vivo* perfusion utilizing human placentae that are obtained immediately after delivery [30–32]. Here, the structure of the cotyledon as a functional unit of the placenta is fully preserved [33], and data obtained are highly predictive of the *in vivo* transfer [34].

We here determined the transplacental transfer of protopine, a major alkaloid in California poppy [35] and several other medicinal plants [15], side by side with compounds known to cross the placental barrier, like antipyrine, citalopram, and diazepam (▶ **Fig. 1**). The effects of protopine on the viability of placental tissue and the production of placental hormones were also investigated.



▶ **Fig. 1** Structures of connectivity control (antipyrine), the selected phytochemical (protopine), and synthetic study compounds (citalopram, diazepam).

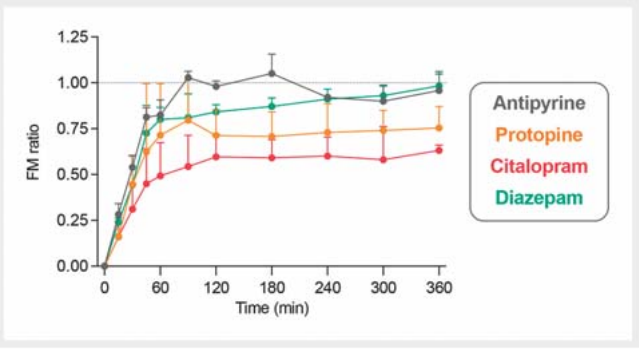


▶ **Fig. 2** *Ex vivo* human placental perfusion profiles of (**a**) citalopram (n = 4), (**c**) diazepam (n = 5), and (**e**) protopine (n = 4, except at t = 300 and 360 min, in which only 2 values are included) with corresponding connectivity control (antipyrine) transfers (**b, d**, and **f**, respectively). Concentrations are expressed as a percentage (%) of initial analyzed concentration in the maternal sample. All values are expressed as mean ± standard deviation (SD). Perfusion profiles with absolute concentrations (ng/mL) can be found in **Fig. 1S** (Supporting Information).

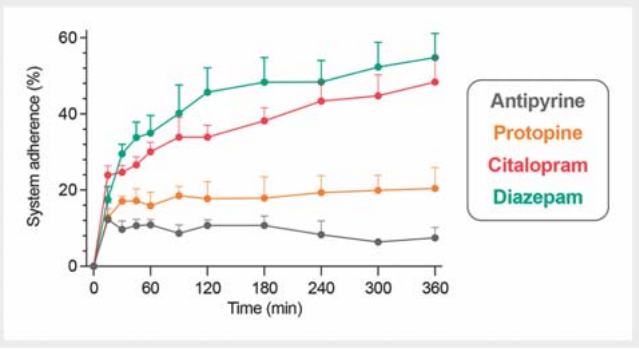
Results

As a first step, the *ex vivo* placental perfusion model – with which we wanted to study the transfer of protopine – was validated with 3 placenta-permeable compounds: antipyrine, citalopram, and diazepam (▶ **Fig. 2**). Antipyrine served as a connectivity (positive) control in all placental perfusions to verify the overlap of the maternal and the fetal side. Citalopram and diazepam crossed the human placental barrier as expected [36, 37]. After approximately 60 min, a steady-state concentration was achieved on the fetal side corresponding to 11% of the initially present citalopram at the maternal side. Thereby, a steady-state concentration of 22% was reached on the maternal side. Concentrations did not change during the following 300 min (▶ **Fig. 2a**). For diazepam, steady-state concentrations were reached after approximately 45 min, with 24% of the initially analyzed concentration and a slow decrease until 360 min (▶ **Fig. 2b**). Antipyrine also readily crossed the placental barrier and reached a steady-state concentration after approximately 120 min (▶ **Fig. 2c** and **d**). These findings aligned with previous work and confirmed that antipyrine was a suitable connectivity control [38].

Protopine was also transferred from the maternal to the fetal circuit. A gradual decrease of protopine in the maternal compartment and a concomitant increase in the fetal compartment were observed. After about 60 min, a steady-state was established in



▶ **Fig. 3** The fetal-maternal concentration ratio (FM ratio; fetal concentration divided by maternal concentration) calculated for each timepoint of protopine (n = 4), citalopram (n = 4), and diazepam (n = 5), in comparison with antipyrine (n = 3) from control perfusions. The FM ratio is 1.0 when the fetal and maternal concentrations are equal. All data are expressed as mean ± standard deviation (SD) of at least 3 independent experiments (except t = 300 and 360 min in case of protopine, and t = 360 min in case of antipyrine, in which only 2 values are included).



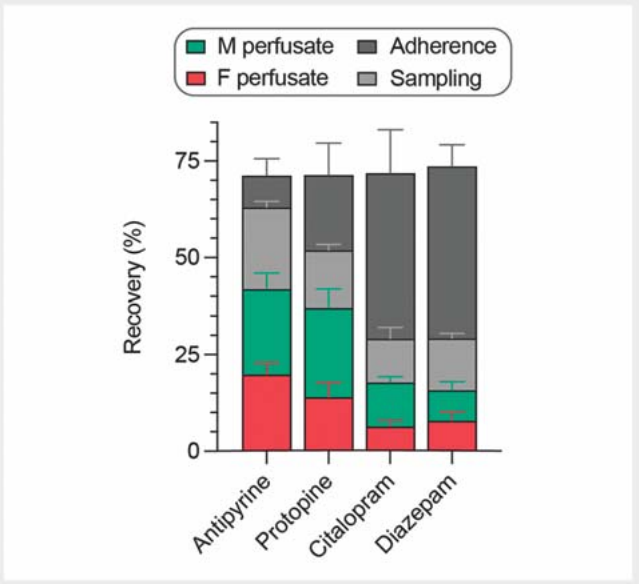
▶ **Fig. 4** Adherence of study compounds from a 360 min system adherence test (circulation of study compounds through an empty perfusion chamber comprising only the maternal circuit). All compounds were tested individually in 3 independent experiments (n = 3) and are expressed as mean ± standard deviation (SD). Displayed is the percentage (%) of compound (of initially analyzed concentration in the maternal sample) that adheres to the equipment: antipyrine (7.4 ± 4.7%), protopine (20.4 ± 9.5%), citalopram (48.4 ± 12.9%), and diazepam (54.8 ± 12.7%) at 360 min.

the 2 circuits, with virtually no change over the remaining 300 min (approximately 27% [maternal] vs. 20% [fetal] of initially analyzed concentration; ▶ **Fig. 2e**). The overlap of maternal and fetal circuits was again confirmed with antipyrine reaching an equilibrium after 120–240 min (▶ **Fig. 2f**). Perfusion profiles with absolute concentrations (ng/mL) can be found in **Fig. 1S** (Supporting Information).

If the fetal-maternal concentration ratio (FM ratio; ▶ **Fig. 3**) of protopine is considered, no concentration equilibrium was apparent in the fetal and maternal compartments at any point of the placental perfusion (FM ratio of 0.75 after 360 min). The profiles of the synthetic compounds are shown for comparison. In citalopram, an equilibrium between fetal and maternal concentrations was never reached (FM ratio of 0.63 after 360 min). As for diazepam, the FM ratio was comparable to that of antipyrine (0.98 vs. 0.95 at 360 min).

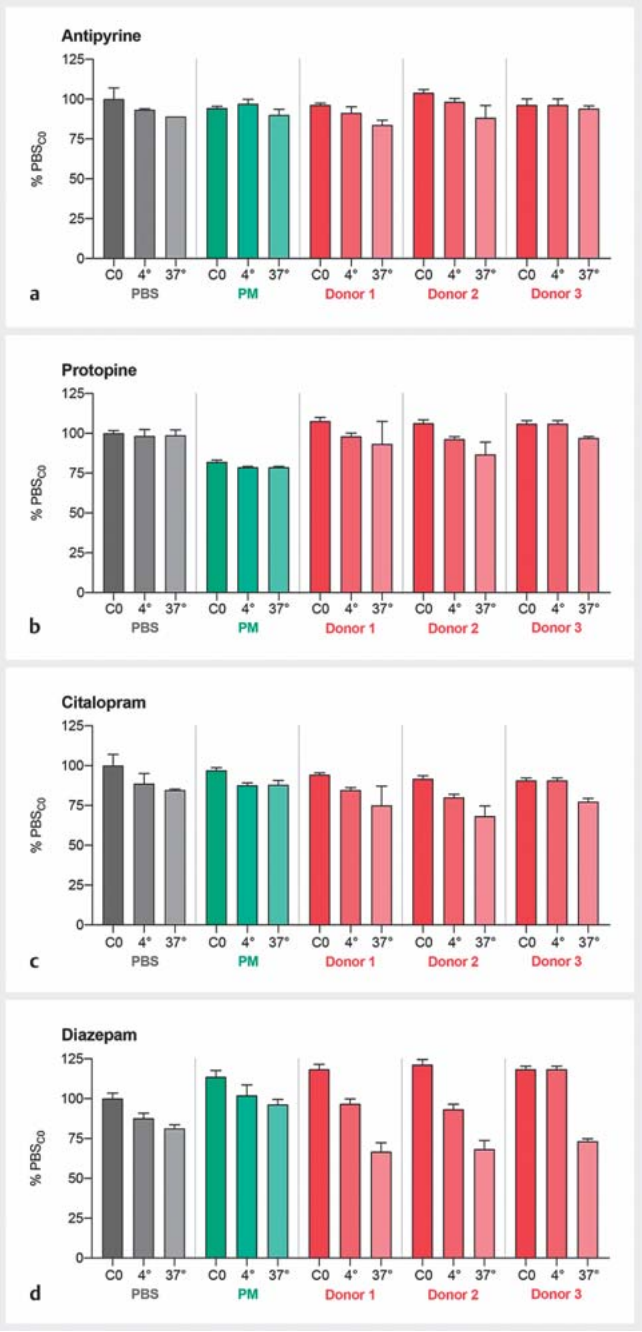
The 360 min system nonspecific adherence tests (empty perfusions; ▶ **Fig. 4**), which were performed without placenta and only in the maternal circuit, revealed that only minor proportions of protopine and the connectivity control antipyrine were lost over 360 min (20.4% and 7.5% of initially analyzed concentration, respectively). The relative amount of citalopram and diazepam which adhered to the perfusion equipment after 360 min, was significantly higher with 48.4% and 54.8%, respectively.

Several aspects must be considered for the recovery calculations (▶ **Fig. 5** and **Table 1S**, Supporting Information) of study compounds during placental perfusions. Looking at the final distribution of the compounds in the 2 compartments (fetal and maternal circuit) after 360 min of the perfusion, we found they all passed the placental barrier and were distributed in the following proportions (fetal vs. maternal): antipyrine (19.8% vs. 22.0%), protopine (14.0% vs. 23.1%), citalopram (6.4% vs. 11.4%), and diazepam (7.9% vs. 7.9%). As shown in **Table 1S** (Supporting Information), 11.4–21.2% of the substance was removed by sampling during perfusion, corresponding to one-sixth to one-third



▶ **Fig. 5** Recovery of study compounds in the human *ex vivo* placental perfusion system, expressed as percentage (%) of initial amount analyzed in the maternal sample at the beginning of the perfusion. The final recovery was calculated as the sum of compound present in fetal and maternal perfusates at the end of a perfusion and the amounts sampled during the perfusion from fetal and maternal perfusates. The loss of compound by binding to the perfusion model was accounted for by the system adherence test (empty perfusion). All data are represented as mean ± standard deviation (SD) of 3 to 5 independent experiments.

of the final recovery. In addition, it is crucial to include the results from the 360 min system adherence test (empty perfusion). While only small losses were seen for antipyrine and protopine, considerable amounts of citalopram and diazepam adhered to



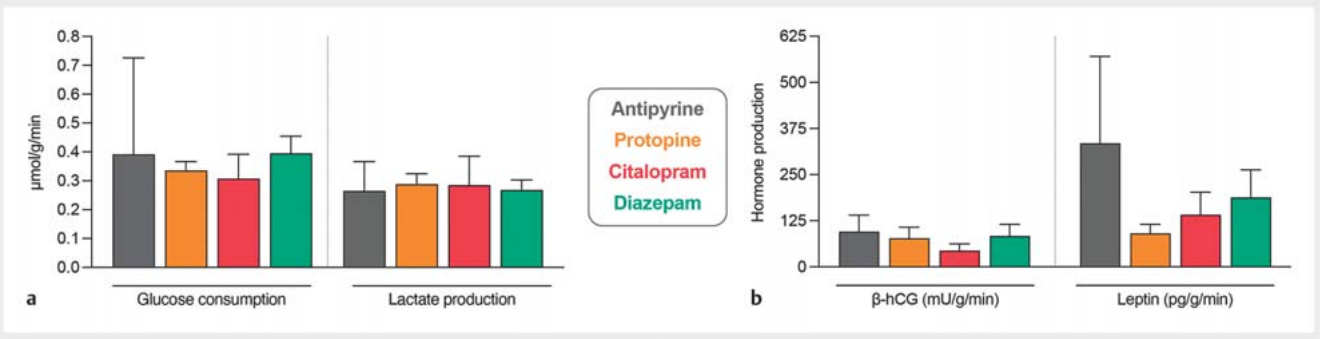
▶ **Fig. 6** Stability data of study compounds (**a**–**d**) expressed as a percentage (%) of the initial concentration (C0) in PBS. The stability test was performed for 360 min at 2 different temperatures (4 °C and 37 °C) and 3 different matrices (PBS, perfusion medium (PM), and placental homogenates from 3 different donors). Differences due to matrix effects were excluded in a separate experiment (see **Fig. 2S**, Supporting Information). Samples were processed via solid phase extraction or protein precipitation before analysis. All data are represented as mean ± standard deviation (SD).

the equipment and tubing after 360 min. Using the system adherence test to assess the final recovery, we obtained the following values for antipyrine (71.2 ± 7.2 %), protopine (71.4 ± 8.6 %), citalopram (71.9 ± 5.2 %), and diazepam (73.6 ± 4.8). Finally, the fraction unbound to homogenates ($f_{u,hom}$) of placental tissue was assessed to account for potential loss of compound in the placenta itself (**Table 2S**, Supporting Information). The $f_{u,hom}$ was equal to 1.0 for antipyrine, followed by protopine (0.48 ± 0.04), citalopram (0.21 ± 0.01), and diazepam (0.09 ± 0.007). Thus, $f_{u,hom}$ followed the pattern of the system adherence test, reflecting the lipophilic nature of the compounds.

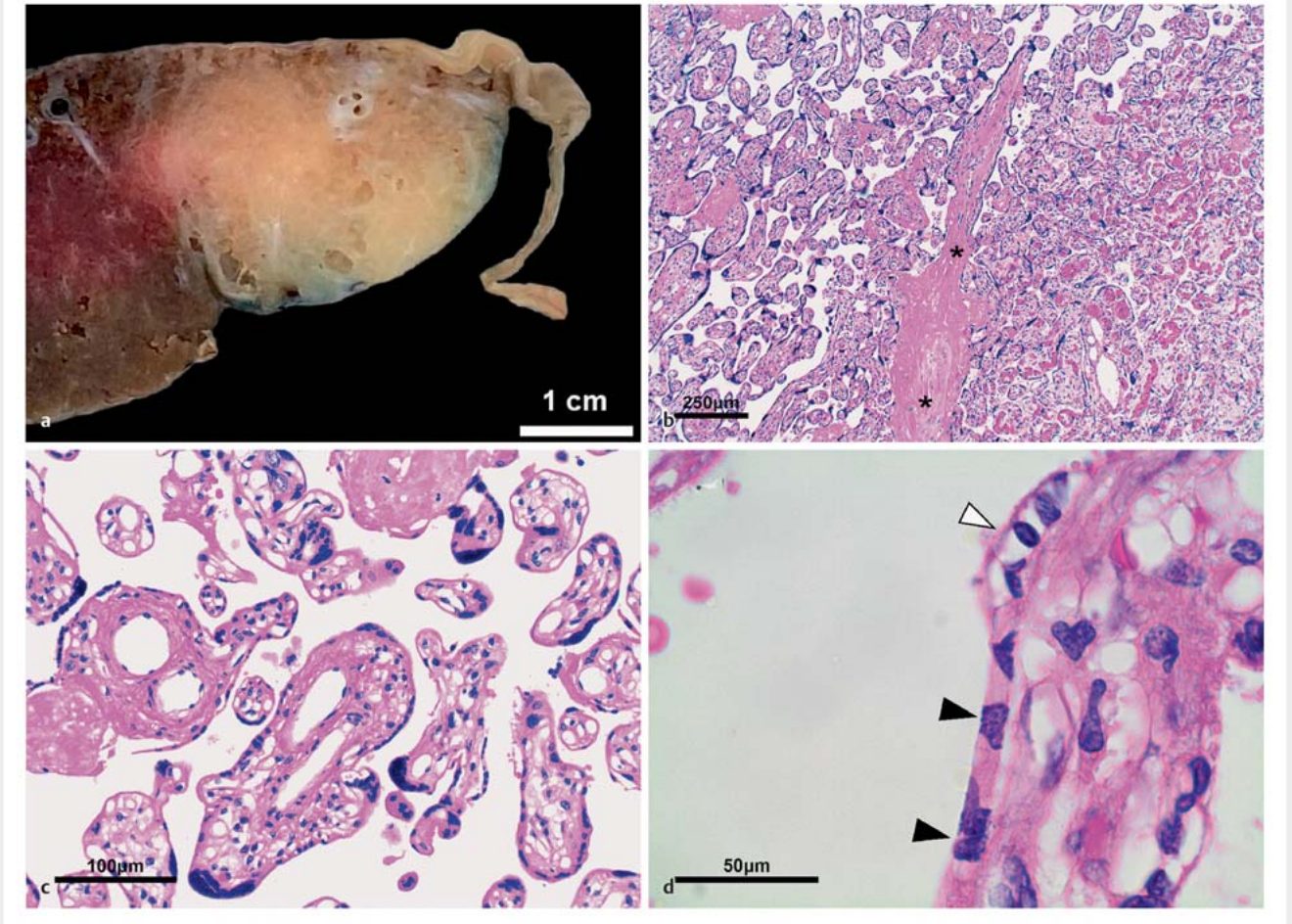
The stability of study compounds was tested over 360 min in 3 different matrices (PBS, perfusion medium (PM), and 3 placental homogenates [Donors 1–3] at 2 temperatures (4 °C, 37 °C). The stability data of antipyrine, and citalopram were very comparable in the 2 matrices PBS and PM, while protopine (in PM) and diazepam (in PBS) were slightly less stable over 360 min at 4 °C and 37 °C (▶ **Fig. 6**). A degradation in homogenate at 37 °C was observed with diazepam (66.7 %, 68.3 %, and 73.3 % respectively). The use of 3 different placental homogenates (donors) resulted in comparable values for all test substances. Differences due to matrix effects were excluded in a separate experiment (see **Fig. 2S**, Supporting Information).

The placental perfusion model can not only be used to characterize the transplacental compound transfer and investigate the possible effects of the compounds on placental viability and hormonal production. All placentae viability and metabolic activity were constant in our case, as neither glucose consumption nor lactate production was affected by the study compounds (▶ **Fig. 7a**). With antipyrine (from control perfusions, **Fig. 3S**, Supporting Information), the total glucose consumption and lactate production during 360 min were 0.39 and 0.27 µmol/g/min, respectively. Perfusions with protopine, citalopram, and diazepam showed comparable values, indicating that they did not impair placental viability and metabolic activity. As an additional measure for placental function, the production of beta-human chorionic gonadotropin (*β*-hCG) and leptin was determined and expressed as the release rate per min and weight of cotyledon (g) (▶ **Fig. 7b**). The tissue of all placentae retained its functionality throughout the *ex vivo* perfusion period. A *β*-hCG production of 95.7 mU/g/min and leptin production of 334.8 pg/g/min were observed in control perfusions with antipyrine (from control perfusions, **Fig. 3S**, Supporting Information). Protopine did not inhibit *β*-hCG and leptin production in a statistically significant way, even though leptin production was somewhat lower in the presence of all study compounds.

To establish the human *ex vivo* placental perfusion model, we introduced a detailed histopathological examination of the perfused tissue. Representative macroscopic and microscopic images of the transition between perfused and nonperfused tissue, and details of the perfused area and an individual villus with an outer layer of trophoblast cells are shown (▶ **Fig. 8**). The most important histopathological criteria for quality control (QC) were: (i) macroscopically, an evident effect of the perfusion on the cotyledon, in contrast to the nonperfused tissue (▶ **Fig. 8a**); microscopically, (ii) a difference between perfused and nonperfused tissue, the latter being characterized by a narrow intervillous space and blood-filled capillaries (▶ **Fig. 8b**); (iii) the perfused area had dilated intervillous space, bloodless capillaries, and regular (mature) villi (▶ **Fig. 8c**); and (iv) the proportion of vacuolated (degenerated) trophoblast cells was between 0–20 % (▶ **Fig. 8d**).



▶ **Fig. 7** Assessment of tissue viability and functionality during the *ex vivo* human placental perfusion. **a** Comparison of glucose consumption and lactate production of the selected phytochemical (protopine) and synthetic compounds (citalopram, diazepam) with antipyrine from control perfusions. Displayed are the changes between beginning and end of the perfusion in fetal and maternal circuits. Normalized by the total perfusion time (min) and perfused cotyledon weight (g). **b** Comparison of beta-human choriogonadotropin (*β*-hCG) and leptin tissue production of perfusions with the selected phytochemical (protopine), synthetic compounds (citalopram, diazepam), and antipyrine (from control perfusions). The net release rate of placental hormones is displayed during the placental perfusion, normalized by the total perfusion time (min) and perfused cotyledon weight (g). All data are represented as mean ± standard deviation (SD) of 3 to 5 independent experiments.



▶ **Fig. 8** Histopathological evaluation of placental tissue as additional quality control. **a** Macroscopic image of a representative placental specimen from the transitional area of nonperfused and perfused tissue (left and right, respectively; scale bar 1 cm). **b** Overview of the perfused vs. nonperfused area (left and right, respectively; scale bar 250 µm). The septum (asterisk) represents the (incomplete) partition separating the cotyledons. The blood-filled capillaries and the narrow intervillous space, which is also partly filled with blood, are clearly visible on the right. **c** Perfused parenchyma showing mostly empty capillaries and empty, dilated intervillous space. Parenchyma shows inconspicuous, regular mature villi (scale bar 100 µm). **d** Close-up of a villus from the perfused area showing vacuolated degenerate and regular trophoblast cells (white and black arrowheads, respectively; scale bar 50 µm).

▶ **Table 1** Detailed histopathological evaluation assessing the microscopic effects of human *ex vivo* placental perfusions with protopine (n = 4), and the damage of placental tissue in perfused areas compared to nonperfused areas.

	Experiment number	Protopine			
		1	2	3	4
Microscopic effects of perfusion (in perfused tissue) in %	Sharp transition perfused/nonperfused	Y	Y	Y	Y
	Emptiness of villous vessels	90	80	95	95
	Blood-filled villous vessels in nonperfused	90	95	100	95
	Dilatated (recognizable) villous vessels	95	50	60	ND
	Intervillous spaces without blood	100	95	95	95
	Blood-filled intervillous space in nonperfused	50	30	5	20
	Dilatated intervillous space	80	40	80	50
	Dilatated intervillous space in nonperfused	30	10	30	40
	Hydropic changes	15	10	5	5
	Hydropic changes in nonperfused	1	0	0	0
Damage of placental tissue (in perfused tissue) in %	Thrombi in villous vessels	1	5	0	5
	If so: in nonperfused too?	1	1	–	1
	Thrombi in vessels of stem villi	0	0	0	0
	If so: in nonperfused too?	–	–	–	–
	Vacuolated trophoblast in villi	0	20	1	1
	If so: in nonperfused too?	–	0	0	0

ND = not determined; Y = yes.

Initial histopathological examinations showed that, in our hands, perfusion was effectively taking place and did not seriously damage the perfused cotyledon. These examinations were then performed after every perfusion experiment with study compounds to demonstrate successful perfusion (including connection of the fetal and maternal perfused area) and assess possible deleterious effects of the compound on the tissue compared to nonperfused tissue.

The histopathological evaluation of the placental specimens showed that the only macroscopically discernible effect of the perfusion was the pale tissue, which was apparent in all placentae. In all cases, the microscopic examination (▶ **Table 1**) of the tissue sections revealed a clear transition between perfused and nonperfused tissue. The villous vessels of the perfused side were ≥ 80 % empty (the nonperfused area was ≥ 90 % blood-filled) and mostly dilated. The intervillous spaces of the perfused tissue were also ≥ 95 % bloodless (the nonperfused area was ≤ 50 % blood-filled) and mostly dilated (40–80 %), in contrast to the nonperfused side (10–40 %). Hydropic villous changes were found more often in perfused (5–15 %) than in nonperfused areas (0–1 %). Histopathological examinations showed that the endothelium in perfused and nonperfused tissue was still viable after 360 min of perfusion. There were also no ruptures of villous vessels or extravasation into villous stroma in perfused and nonperfused areas. In addition, no thrombi could be detected in vessels of stem villi. The percentage of thrombi in villous vessels (0 % or 1 %) was the same in perfused and control tissue (nonperfused), whereas 2 cotyledons perfused with protopine showed a slightly higher proportion of villous thrombi compared to control (5 % vs. 1 %). Trophoblast vacuolization in the perfused areas occurred in a small proportion of 0–20 %. Overall, protopine did not cause apparent damage of placental tissue according to the assessment of endothelium, vascular rupture, thrombi, and trophoblast vacuolization. In addition, no signs of inflammation were found in any of the perfused areas of the placentae examined, as neither bacteria nor neutrophils were present in the villous vessels and intervillous spaces. The assessment of global placental pathology was also inconspicuous, with no signs of fetal or maternal malperfusion, an absence of villous immaturity, chronic/acute villitis, chronic deciduitis and chorioamnionitis, and no bacteria in the nonperfused area of the placenta.

Discussion

The present data show that protopine was rapidly transferred from the maternal to the fetal circuit, and no evidence for metabolization was found. However, the FM ratio of protopine was lower than that of antipyrine (0.75 vs. 0.96), and no equilibrium between maternal and fetal concentrations was reached. This finding was similar to the results obtained with citalopram. Whether the absence of an equilibrium is due to active transplacental transport of protopine deserves further investigation. In our experiments, the transplacental transfer of antipyrine, citalo-

pram, and diazepam was comparable with previously reported data [36–38].

The use of placental perfusion and U(H)PLC-MS/MS methods are strengths of the present work. Placental perfusion is the gold standard when studying the transfer of compounds from the mother to the fetus. A limitation of this model is that substance transfer at term may be overestimated compared to that in the premature placenta of early pregnancy [28]; in addition, the model cannot mimic the mother's drug absorption, distribution, metabolism, and excretion. Only U(H)PLC-MS/MS methods to detect very low concentrations enabled the work on this project, since clinically relevant concentrations of phytochemicals from (multicomponent) extracts are known/expected to be very low.

Ex vivo placental perfusions allow determining the transfer of study compounds and assessing placental viability and function. Control perfusions with antipyrine showed similar values for the placental viability (glucose consumption, lactate production) and functionality (β-hCG and leptin production) as previously reported for this model [32]. None of the study compounds altered the glucose consumption, lactate production, and β-hCG accumulation, and only a statistically nonsignificant decrease in leptin production was observed. These results indicated that none of the compounds impaired placental performance. The histopathological evaluation of perfused tissues (cotyledons) was in line with these results. No pathological findings were observed, and all placental specimens showed only unremarkable, age-appropriate parenchymal maturation. Trophoblastic vacuolization of villi was the only perfusion-induced change observed. Despite the ischaemic periods up to 60 min, no increase in damage of placental tissue could be observed with time (▶ **Table 1** and **Table 3S**, Supporting Information). Neither the number of thrombi in villous vessels nor the vacuolization of trophoblasts in villi was increased.

It was crucial to calculate the recovery of study compounds in the best possible way to validate the findings. The amounts in the fetal and maternal compartments, the amounts removed by sampling, and the loss due to adsorption in the maternal circuit could be considered. Study compounds may also be taken up by cells/membranes and may be metabolized by placental enzymes. To minimize adsorption to the perfusion system, we used only tubes recommended for pharmaceutical and medical applications, and we shortened the tubing in the model. Moreover, in placental homogenates, fraction unbound, and stability of compounds were determined. Nevertheless, the calculated recoveries are likely to be underestimated, given that adsorption in the fetal circuit could not be measured.

To further assess safety of California poppy during pregnancy, transplacental passage of additional phytochemicals have to be investigated, together with testing in additional models. We are currently using a range of *in vitro* assays to characterize cytotoxic and genotoxic effects, and the influence on metabolic and differentiation processes of whole extracts [39] and protopine (Spiess et al., manuscript under review). From a safety perspective, these results will be particularly relevant for compounds that can cross the placental barrier and, therefore, have access to the fetal circulation, as is the case for protopine. In addition, we are investigating the intestinal and hepatic metabolization of several phytochemicals.

The human placental *ex vivo* perfusion model was successfully implemented and used for the first time with a phytochemical. The *ex vivo* placental perfusion model will now be used also for transport studies with relevant phytochemicals in other medicinal plants used for the treatment of mild NMDs in pregnancy.

Materials and Methods

Chemicals, reagents, and study compounds

All solvents were of U(H)PLC grade. Merck KGaA supplied MeCN, and MeOH was purchased from Reuss-Chemie AG. Purified water was obtained from a Milli-Q integral water purification system.

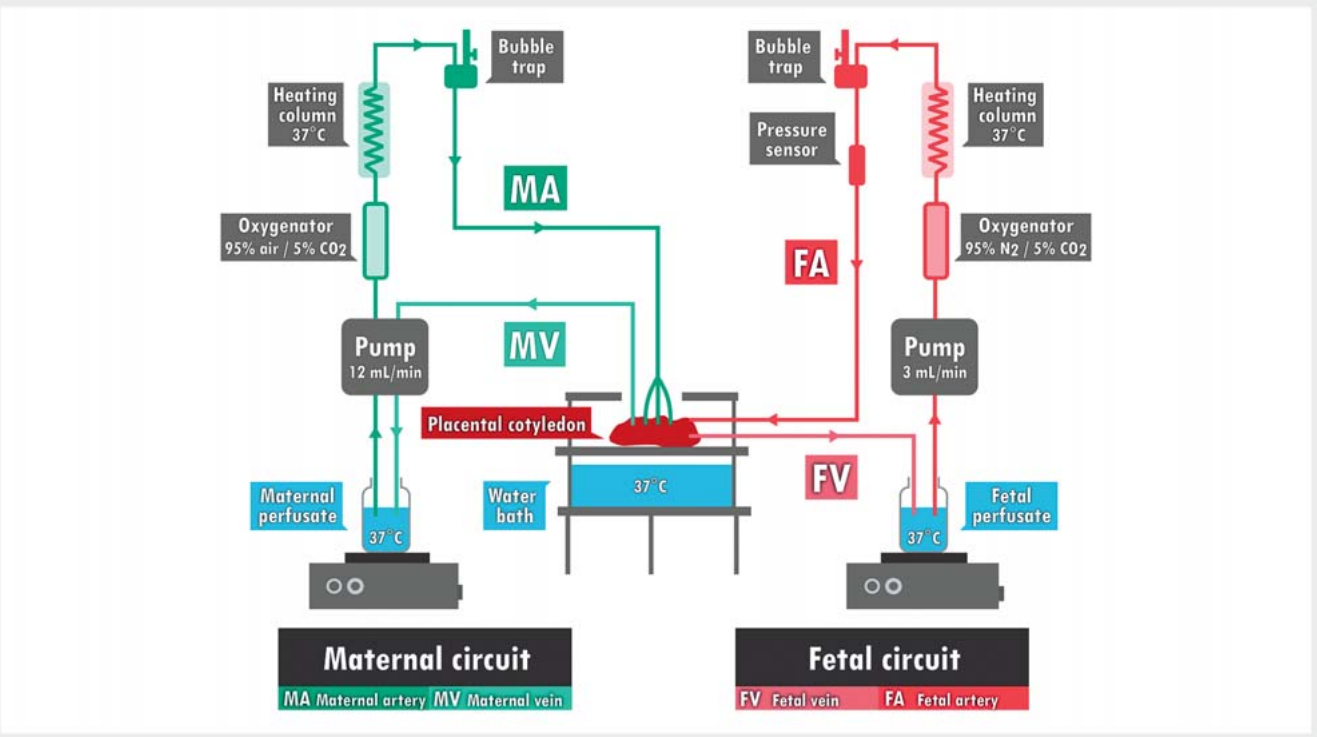
Scharlau supplied DMSO, and formic acid was from BioSolve. Antipyrine and BSA were purchased from Sigma-Aldrich and antipyrine-D3 from HPC Standards GmbH. Protopine HCl was purchased from Extrasynthese SAS, and verapamil HCl from Sigma-Aldrich. Citalopram HBr, diazepam, and diazepam-D5 were purchased from Lipomed AG, and citalopram-D4 HBr was obtained from CDN Isotopes.

Ex vivo human placental perfusion

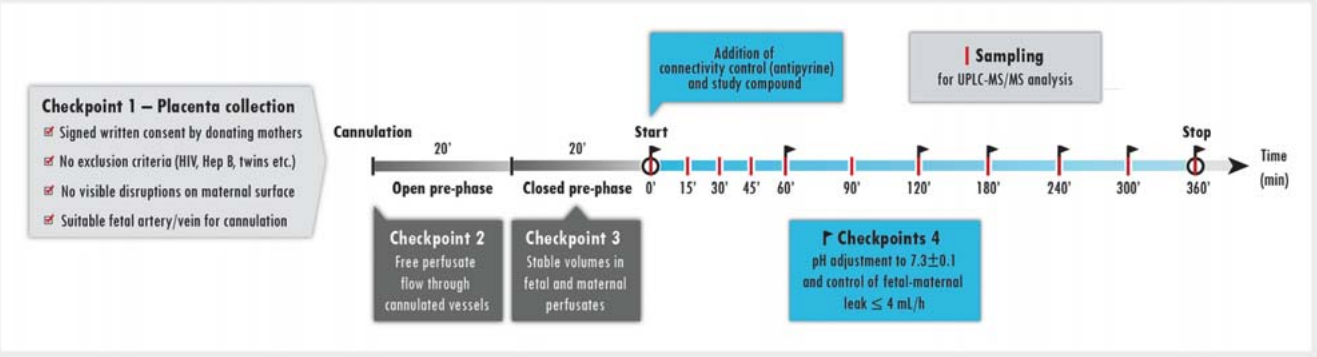
Placentae collection. Placentae were collected in collaboration with the Department of Obstetrics of the University Hospital of Zurich, Switzerland. Only placentae from women undergoing elective caesarean section from uncomplicated term pregnancies (37–41 wk) were considered. Each pregnant woman signed informed written consent before delivery for the use of placentae for research. This procedure (including consent form) was approved by the Ethics Committee of the Canton of Zurich (KEK-StV73 Nr. 07/07; March 21, 2007). Exclusion criteria included: twin and/or complicated pregnancy, smoking, substance abuse, and patients positive for HIV, HBV and SARS-CoV-2.

Equipment and experimental procedure of perfusion. A slightly modified *ex vivo* human placental perfusion model [32, 38] was used to study the transfer of the study compounds across the placental barrier (▶ **Fig. 9**). A cotyledon of the placenta (lobule) was perfused with 2 reconstructed circuits representing the maternal and fetal sides. Both sides consisted of an artery transporting the perfusate to the cotyledon and a vein that returns the perfusate to the original reservoir. Two heating magnetic stirrers were added to ensure physiological conditions and to prevent uneven distribution of study compounds.

The time course of a perfusion experiment, including the preparatory phases, checkpoints and samplings is shown in ▶ **Fig. 10**. After obtaining a suitable placenta (checkpoint 1), the fetal artery and associated vein of a selected cotyledon were cannulated and mounted in a perfusion chamber within 60 min after delivery. The selection of a suitable cotyledon for perfusion was based on a thorough visual examination of the villous structures and associated fetal vessels. Cotyledons with a ragged maternal surface (visible disruptions; macroscopic tissue trauma), evidence of basal plate fibrin deposition (on the maternal surface), suspected placental infarction, or too little fetal membrane (on the disk of the placenta) were not considered for perfusion. The chorionic artery was cannulated (⌀ 1.2 mm cannula) first, following the chorionic vein cannulation (⌀ 1.5–1.8 mm cannula). A surgical



▶ **Fig. 9** Placental perfusion setup consisting of maternal (left) and fetal (right) circuit. Both perfusates are placed on magnetic stirring and heating devices and transported to the placental cotyledon (lobule) via arteries with the aid of peristaltic pumps operated at different flow rates (maternal: 12 mL/min; fetal: 3 mL/min). Flow heaters (heating columns) and a water bath below the perfusion chamber keep the temperature at 37 °C. On the way to the placental cotyledon, the perfusates are gassed by oxygenators with non-identical compositions (maternal: 95% air/5% CO_2; fetal: 95% N_2/5% CO_2) and freed from any air bubbles by bubble traps. A sensor in the fetal artery records the pressure (and temperature). Two veins return the perfusates to the corresponding reservoirs.



▶ **Fig. 10** Overview of the *ex vivo* human placental perfusion with checkpoints for a successful experiment. Checkpoint 1 involved obtaining a suitable placenta, written informed consent from the donor, and ensuring that no exclusion criteria were present. The quality of placental tissue and presence of fetal vessels suitable for cannulation were checked. After cannulation, it is important to verify free flow of perfusion medium (PM) through the fetal vessels (checkpoint 2), leading to a 20 min open preliminary (pre-) phase (nonrecirculating), followed by a 20 min closed pre-phase (recirculating). During the latter, the volumes of maternal and fetal perfusates have to remain stable in order to begin the main experiment (checkpoint 3). The main experiment started with circulating PM containing the connectivity control (antipyrine) and study compound at the desired concentration (O denoting the experiment's beginning and end). At defined timepoints (0, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min), samples were taken from the maternal and fetal reservoir for analysis. At the beginning of the experiment and after each hour, the pH and the fetal-maternal (FM) leak were measured.

suture (orange or green PremiCron HR17, USP3/0, B. Braun Medical AG) was used to fix the cannulas. The experiment was performed using PM (modified composition [32] using cell culture medium 199 from Sigma-Aldrich) circulating through the fetal and maternal circuit using digitally controlled peristaltic pumps (Ismatec) at a rate of 3 and 12 mL/min, respectively. The tubing consisted of a fetal artery (∅ 1.52 mm) and maternal artery tube (∅ 2.06 mm), a maternal vein tube (∅ 2.29 mm), and connecting tubes (∅ 1.60 mm, all PharMed Ismaprene from Ismatec). The fetal perfusate was gassed with 95% N_2/5% CO_2 throughout the perfusion and the maternal perfusate with 95% air/5% CO_2 instead. The perfusion included a 20 min open preliminary (pre-) phase with nonrecirculating PM (with discarded venous outflow) to allow the placental tissue to recover from the ischemic period after the delivery and to flush out the remaining blood from the villous vasculature and intervillous space (checkpoint 2). In the following recirculating closed pre-phase (20 min; with the venous outflow leading back to the corresponding reservoir), the perfusate volumes on the maternal and fetal sides were monitored. Stability of volumes (50 mL PM in each reservoir) ensured the integrity of the circuits and the absence of leaking in the fetal-to-maternal circuit (checkpoint 3). The main experiment was then initiated by replacing the fetal and maternal perfusate simultaneously with equal starting volumes of 100 mL fresh PM, with the addition of the study compound and antipyrine (as a connectivity control) to the perfusate of the maternal circuit, both at a final concentration of 5 µM (≙ 941 ng/mL antipyrine, 1767 ng/mL protopine, 1622 ng/mL citalopram, and 1424 ng/mL diazepam). In both reservoirs, the pH was adjusted to a physiological range (7.3 ± 0.1) at the beginning of the experiment (and adjusted after every hour of perfusion), and the fetal-maternal (FM) leak was not allowed to exceed a value of 4 mL/h (checkpoint 4). Samples (3–4 mL) for analysis were taken at defined timepoints over a 360 min period (0, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min). Samples were stored at − 80 °C for bioanalytical analysis in 0.8 mL glass micro-inserts (VWR) immediately after centrifugation at 800 rpm/1837 rcf (Centrifuge Sigma 6–16; 4 °C, 10 min) to remove residual erythrocytes. Additional QC measures included a fetal perfusion pressure ≤ 70 mmHg throughout the perfusion and an antipyrine equilibrium between fetal and maternal circuit after 240–360 min. For an overview of the experimental conditions and characteristics of the placentae used see **Table 3S** (Supporting Information).

Fetal capillary integrity marker analysis. For establishing and validating the *ex vivo* placental perfusion model, initial experiments were performed using fluorescein isothiocyanate (FITC)-dextran (40 kDa, Sigma-Aldrich) as a test substance for assessing the integrity of fetal capillaries (**Fig. 4S**, Supporting Information). FITC-dextran was added to the fetal circuit at a final concentration of 200 µg/mL. The concentration in the fetal and maternal samples was detected by a Cytation 3 fluorescence microplate reader (BioTek Instruments; excitation wavelength 490 nm; emission wavelength 520 nm). The samples (undiluted) were added to black Nunc MaxiSorp microtiter plates.

Perfusion system adherence test (empty perfusion). Before starting the perfusions with human placentae, a system adherence test (circuit of study compounds through an empty perfusion chamber comprising only the maternal circuit) was performed for a total of 360 min. This test was used to assess the dissolution and adherence of new, yet unknown, compounds to the perfusion equipment and mainly to the tubing system to evaluate the final recovery better. Study compounds were, therefore, directly dissolved in PM at a final concentration of 5 µM. All study compounds were tested individually in 3 independent experiments (n = 3).

Viability and functionality of placental tissue. We measured glucose and lactate concentration in fetal and maternal samples at the beginning and end of every perfusion. This was done to determine the glucose consumption and lactate production throughout a perfusion as indicators of tissue viability and metabolic activity using an automated blood gas system (ABL800 FLEX). The production of 2 placental hormones – β-hCG and leptin – was monitored to assess tissue functionality *ex vivo* by standard ELISA as described previously by Malek et al. [40, 41]. The only deviation following the protocol was the use of a dilution solution consisting of 1% BSA instead of 2% BSA.

Histopathological evaluation. Each placental specimen was pathologically examined as an additional QC. For this purpose, representative tissue sections – each from the perfused and nonperfused placental portion, and from the transitional area – were removed immediately after each perfusion, fixed in 4% paraformaldehyde for at least 24 h, and then processed according to the standards of routine histopathological diagnosis of the Department of Pathology and Molecular Pathology (University Hospital of Zurich). Briefly, the fixed tissue sections were embedded in paraffin, cut into 2–3 µm thick sections, then stained with standard hematoxylin and eosin stain and with a modified Gram stain (according to Braun-Brenn). The latter was used to test for bacterial contamination in the perfused area [42]. Tissue from the nonperfused specimens was examined for general placental pathologies described in routine diagnostics [43, 44]. The quality of perfusion was correlated based on the blood void and width of the intervillous (maternal) space and fetal blood vessels in the chorionic villi, with particular attention to the presence of intravascular thrombi. To test whether tissue damage might have occurred due to perfusion, we sought and compared general signs of degeneration such as vacuolization of the cytotrophoblast, the viability of the villous vascular endothelium, and the formation of hydropic villous changes with the tissue condition of the nonperfused area. All microscopic effects studied and damage to placental tissue in the perfused area are reported in relative amounts (% of total).

LC-MS/MS analysis

Instrument and chromatographic conditions. U(H)PLC-MS/MS analyses were performed on an Agilent 6460 Triple Quadrupole MS system connected to a 1290 Infinity LC system consisting of a binary capillary pump G4220A, column oven G1316C, and multisampler G7167B. Quantitative analysis by MS/MS was performed with electrospray ionization (ESI) in MRM mode. Desolvation and nebulization gas was nitrogen. MS/MS data were analyzed with Agilent MassHunter Workstation software version B.07.00. The temperature of the autosampler was 10 °C. An Acquity UPLC HSS T3 column (100 mm × 2.1 mm; 1.8 µm) (Waters Corp.) was used

for separation of the analyte and the internal standard (IS), except for diazepam and its IS diazepam-D5, where a Kinetex column (100 mm × 2.1 mm; 1.7 µm) (Phenomenex) was used. Analysis of citalopram was performed on an Acquity UPLC system consisting of a binary pump, autosampler, column heater, which was connected to an Acquity TQD (all Waters Corp.). Desolvation and nebulization gas was nitrogen. The autosampler temperature was set at 10 °C, and the column temperature at 45 °C. MS/MS data were analyzed with MassLynx software version 4.1. U(H)PLC gradients, internal standards, and MRM transitions can be found in **Table 4S** and **5S** (Supporting Information).

Standards and stock solutions. Stock solutions (SS) of the analytes and the ISs were prepared at 0.2–1 mg/mL. Working solutions (WS1) of the analytes (50 µg/mL for antipyrine, and 100 µg/mL for all other compounds in DMSO) and of the ISs (50 µg/mL in MeOH) were obtained by dilution of the corresponding SSs. Calibration samples (Cals) of the analytes within the range of 5–500 ng/mL (antipyrine), 5–250 ng/mL (protopine), 10–1000 ng/mL (citalopram HBr and diazepam), and QCs at low (QCL), medium (QCM), and high (QCH) levels were obtained from serial dilutions of the WS in the corresponding matrix (PM). The concentrations of the QCs were defined as (i) 3-fold the lowest concentration for QCL; (ii) the highest concentration divided by 2 for QCM; and (iii) 80 % of the highest concentration for QCH. All SSs were stored at − 80 °C. Cals and QCs were freshly prepared before analysis. Before each experiment, a second working solution (WS2) of the IS was prepared by further diluting the WS1 in MeOH. Details for the calibration curves can be found in **Fig. 5S–8S** (Supporting Information) and in **Tables 6S–9S** (Supporting Information).

Sample extraction in placental perfusion medium for antipyrine, diazepam, citalopram, and protopine. To 200 µL of the analyte in the PM were added 100 µL of the IS, 200 µL BSA (60 g/L), and 800 µL ice cold MeCN (1000 µL for antipyrine). The mixture was briefly vortexed at room temperature on an Eppendorf Thermomixer (1400 rpm) and finally centrifuged for 20 min at 13 200 rpm/16 100 rcf at 10 °C (Centrifuge 5415R, Eppendorf). A total of 1100 µL (1300 µL for antipyrine) supernatant was collected and transferred into a 96-deepwell plate (96-DPW) and dried under nitrogen gas flow (Evaporex EVX-96, Apricot Designs). Reconstitution was done with 200 µL of 65 % mobile phase A (purified water with 5 % MeCN and 0.1 % formic acid; in the case of diazepam water and 0.1 % formic acid only) and 35 % mobile phase B (MeCN with 0.1 % formic acid) followed by 45 min shaking on the Eppendorf MixMate. The injection was done in full loop mode (2 µL) from the 96-DWP.

Method qualification. The bioanalytical fit-for-purpose methods had been developed and qualified only based on some validation tests (within- and between-series imprecision and inaccuracy, as well as carry-over had been assessed to validate the methods), following the current guidelines for industry [45, 46].

Within- and between-series imprecision and inaccuracy. Six replicates of 5 QCs (LLOQ [lower limit of quantification: 5 ng/mL], QCL, QCM, QCH, ULOQ [upper limit of quantification: 500 ng/mL]) were processed and injected into the U(H)PLC-MS/MS. Three validation runs on 3 different days were performed to ensure reproducibility. In each run, the imprecision (CV%) of each QC series had to be below 15 % (20 % for LLOQ) within the series. The inaccuracy (RE%) had to be within ± 15 % of the nominal values (± 20 % at the LLOQ). After these 3 runs, CV% and RE% were calculated between the series by determining the overall mean ± standard deviation (SD) for each QC level. The acceptance criteria were the same as for within-series acceptance criteria. Additional information on quality control can be found in **Tables 10S–13S** (Supporting Information).

Carry-over. The carry-over of analyte and IS in each analytical run was determined by injecting a blank sample immediately after ULOQ in both sets of calibrators. The mean carry-over in the blank sample from the 2 calibrators sets should not exceed 20 % of the signal of the LLOQ for the analyte and 5 % for the IS [46]. Details of the carry-over assessment of all study compounds can be found in **Tables 14S–17S** (Supporting Information).

Recovery/mass balance of study compounds in the placental perfusion system

The final recovery of each study compound after the perfusion was calculated with the following equations (Eqs. 1 and 2):

$$\text{Final recovery } (\%) = \frac{\left[C_{M,t_{end}} \times V_{M,t_{end}} + C_{F,t_{end}} \times V_{F,t_{end}} + \sum_{t=t_0}^{t_{end}} (C_{S,t} \times V_{S,t})\right]}{C_{M,t_0} \times V_{M,t_0}} \times \frac{100}{100 + (100 - EP)} \quad (1)$$

$$\text{where EP} = \frac{\left[C_{EP,t_{end}} \times V_{EP,t_{end}} + \sum_{t=t_0}^{t_{end}} (C_{S,t} \times V_{S,t})\right]}{C_{EP,t_0} \times V_{EP,t_0}} \times 100 \quad (2)$$

in which $C_{M/F/S}$ is maternal/fetal/sample concentration (ng/mL), $V_{M/F/S}$ is maternal/fetal/sample volume (mL), t_0 and t_{end} are the beginning and end of the perfusion. The final recovery (%) is the sum of amount of study compound in maternal and fetal perfusates at the end of a perfusion, and samples (S) collected in relation to the initial amount of study compound measured in the maternal perfusate, including the mean amount adhered from 3 system adherence tests (empty perfusion, EP, in %).

Fraction unbound and stability assay in placental homogenate

Preparation of placental homogenate. Placental homogenates were prepared on a Precellys 24 Tissue Homogenizer (cycle: 5000 rpm, 2 × 20 sec) in 2 mL tubes containing 1.4 mm zirconium oxide beads (Precellys). To 1 g placental tissue, 4 mL PBS (without Ca^{2+} or Mg^{2+}; Dominique Dutscher) were added, resulting in a 5-fold dilution (v/w). After homogenization, the tubes were centrifuged for 5 min at 4 °C (1000 rpm), and the supernatant was collected. Samples were kept on ice throughout the whole procedure.

Determination of fraction unbound in placental homogenate. Fraction unbound was determined by membrane dialysis on a RED device (ThermoFisher) with membranes of a 6–8 kDA molecular weight cut-off. A 100-fold concentrated DMSO SS of test compounds was added to the placental homogenates, yield-

ing a final concentration of 2 µM of compound and 1 % DMSO. According to manufacturer's instructions, 200 µL of the spiked homogenates were added to the donor chamber, and 350 µL of blank buffer were added to the receiver chamber. Samples were collected after equilibration (240 min on an orbital shaker at 600 rpm, 37 °C). Samples were analyzed by U(H)PLC-MS/MS. Fraction unbound was calculated as follows [47]:

$$\text{Diluted } f_{u,d} = \frac{\text{Receiver Area Ratio}}{\text{Donor Area Ratio}} \quad (3)$$

and

$$\text{Undiluted } f_u = \frac{1/D}{(1/f_{u,d} - 1) + 1/D} \quad (4)$$

where D is the dilution factor of 5 as stated above.

Stability of compounds in placental homogenate. The stability of the compounds was assessed over 360 min (to match the time of placental perfusion experiments) in PBS, PM, and placental homogenates. Homogenates spiked with study compounds were prepared as described above. After compound spiking in the different matrixes, samples were either immediately processed for U(H)PLC-MS/MS analysis (C0) or kept at 4 °C and 37 °C for 360 min on an orbital shaker (600 rpm) before processing for U(H)PLC-MS/MS analysis. Stability was expressed as follows [47]:

$$\text{Stability as \% remaining} = \frac{\text{Area Ratio at 4°C or 37°C at 6h}}{\text{Area Ratio at C0}} \times 100\% \quad (5)$$

Data processing and calculations

Concentrations in placental perfusion profiles (▶ **Fig. 2**) and system adherence (▶ **Fig. 4**) are expressed as a percentage (%) of initial analyzed concentration in the maternal sample at the beginning of the perfusion. Note that the recovery values were calculated differently (see Eqs. 1 and 2).

The FM ratio (Eq. 6; ▶ **Fig. 3**) was calculated for each point and plotted against the perfusion time (min). Glucose consumption (Eq. 7) and lactate production (Eq. 8) are displayed as the sum of changes (from the perfusion beginning [t_0] to the end [t_{end}]) of total content (µmol) in both fetal and maternal circuits, normalized by the total perfusion time (min) and perfused cotyledon weight (W_{cot}; g). The net release rate of placental hormones β-hCG (mU) and leptin (pg) (Eq. 9) during the placental perfusion was normalized by the total perfusion time (min) and perfused cotyledon weight (g) as well.

$$\text{FM ratio, t} = \frac{C_{F,t}}{C_{M,t}} \quad (6)$$

$$\text{Glucose consumption} = \frac{(C_{M,t_0} \times V_{M,t_0} - C_{M,t_{end}} \times V_{M,t_{end}}) + (C_{F,t_0} \times V_{F,t_0} - C_{F,t_{end}} \times V_{F,t_{end}})}{t_{end} \times W_{cot}} \quad (7)$$

$$\text{Lactate production} = \frac{(C_{M,t_{end}} \times V_{M,t_{end}} - C_{M,t_0} \times V_{M,t_0}) + (C_{F,t_{end}} \times V_{F,t_{end}} - C_{F,t_0} \times V_{F,t_0})}{t_{end} \times W_{cot}} \quad (8)$$

$$\text{Hormone production} = \frac{C_{M,t_{end}} \times V_{M,t_{end}} + C_{F,t_{end}} \times V_{F,t_{end}}}{t_{end} \times W_{cot}} \quad (9)$$

Statistical data analysis

For glucose consumption, lactate production, β-hCG, and leptin production, multiple group comparisons were performed using the Brown-Forsythe and Welch ANOVA tests, followed by the Dunnett's T3 multiple comparisons posthoc test (with individual variances computed for each comparison) with GraphPad Prism (version 9.1.0 for macOS; GraphPad Software). Data are expressed as mean ± SD of at least 3 independent experiments (if not otherwise indicated). Probability values $^*p \le 0.05$ were considered statistically significant from the control group.

Supporting Information

Perfusion profiles of all study compounds with absolute concentrations (ng/mL), compound recoveries, fraction unbound of compounds to the placental homogenate, homogenate matrix effects, perfusion profile of antipyrine from control perfusions, characteristics of placentae used, data from individual perfusions in detail, assessment of the suitability of a fetal capillary integrity marker, and details on the U(H)PLC-MS/MS bioanalytical methods are available as Supporting Information.

Contributors' Statement

APSW, MH, and OP designed the study. DS established, validated, and conducted the placental perfusion experiments, performed data analysis/interpretation, and wrote the first complete version of the manuscript under the supervision of APSW. VFA and AC developed and validated the bioanalytical methods, and VFA performed all analyses. MO and AT supervised method development, and AT performed stability testing and determination of fraction unbound. MR performed the histopathological examinations. ED was assisting in placental perfusions with protopine. All authors were involved in data interpretation and reviewing of the manuscript. All authors agreed with the final version.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Chapter 4 – Supporting Information

Placental passage of protopine in an *ex vivo* human perfusion system

Deborah Spiess[*], Vanessa Fabienne Abegg[*], Antoine Chauveau, Andrea Treyer, Michael Reinehr, Mouhssin Oufir, Elisa Duong, Olivier Potterat, Matthias Hamburger, Ana Paula Simões-Wüst

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[*] *These authors contributed equally to the work and should be considered as joint first authors*

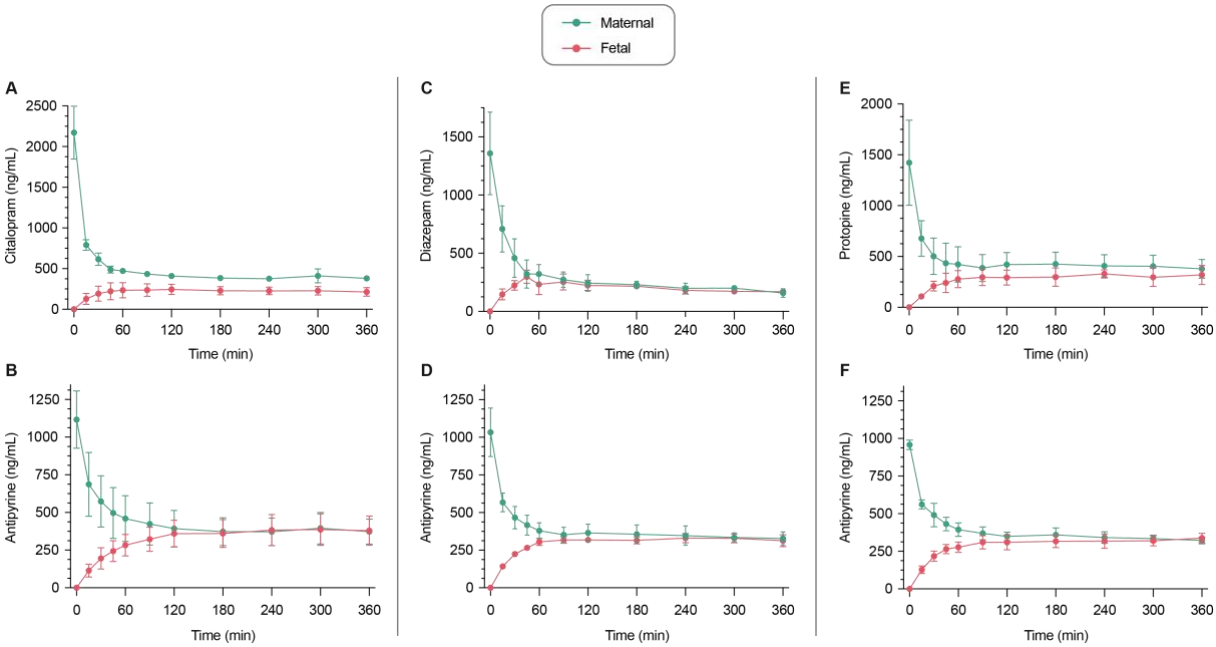


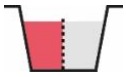
Figure 1S. *Ex vivo* human placental perfusion profiles of citalopram **(A)**, diazepam **(C)**, and protopine **(E)** with corresponding connectivity control (antipyrine) transfers (**B**, **D** and **H**, respectively). Concentrations are expressed as absolute concentrations in ng/mL (● maternal and ● fetal). All values are expressed as mean ± standard deviation (SD) of 3 independent experiments.

Table 1S. Recovery of study compounds after 360 min of *ex vivo* human placental perfusion in relative amounts (% found) ± standard deviation (SD) in different compartments.

	Adherence (% lost)	M perfusate (% found)	F perfusate (% found)	Sampling (% found)	Final recovery* (% found)
Antipyrine	8.2 ± 4.3	22.0 ± 4.2	19.8 ± 3.1	21.2 ± 1.5	71.2 ± 7.2
Protopine	19.5 ± 8.3	23.1 ± 4.8	14.0 ± 3.7	14.8 ± 1.5	71.4 ± 8.6
Citalopram	42.7 ± 11.3	11.4 ± 1.5	6.4 ± 1.5	11.4 ± 2.9	71.9 ± 5.2
Diazepam	44.4 ± 5.6	7.9 ± 2.2	7.9 ± 2.3	13.4 ± 1.3	73.6 ± 4.8

M = maternal; F = fetal; * sum of compound present in fetal and maternal perfusates at the end of a perfusion, the amount sampled during the perfusion (sampling from fetal and maternal perfusates), and adherence of compounds by binding to the perfusion model, determined by the system adherence test (empty perfusion).

Table 2S. Fraction unbound ($f_{u,hom}$) of study compounds to the placental homogenate determined by rapid equilibrium membrane dialysis. The homogenates (from 3 different donors) were each spiked with the compounds and added to the donor chamber. The receiver chamber consisted of blank buffer (PBS). Samples were collected after equilibration (240 min at 37°C, 600 rpm) and were analyzed by U(H)PLC-MS/MS. A dilution factor of 4 was applied to calculate the undiluted fraction unbound.

	Homogenate – PBS  Fraction unbound ($f_{u,hom}$)
Antipyrine	1.00
Protopine	0.48 ± 0.04
Citalopram	0.21 ± 0.01
Diazepam	0.09 ± 0.007

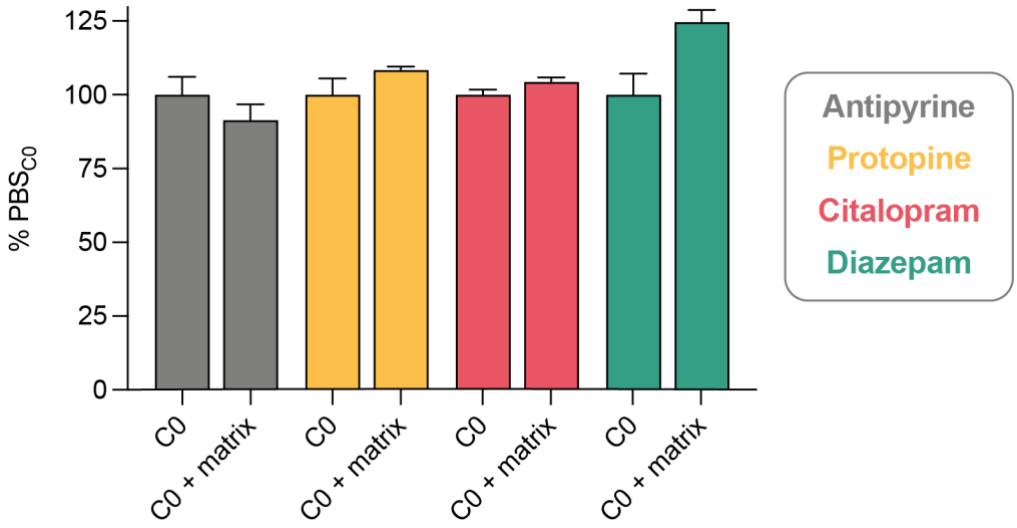


Figure 2S. Matrix effects by the homogenate on the substances antipyrine, protopine, citalopram, and diazepam. The compounds were spiked into PBS and then diluted with either an equal volume of PBS (C0) or an equal volume of tissue homogenate (C0 + matrix). The samples were processed for LC-MS analysis within 1-3 min after the dilution to avoid interference of any stability issues. All values are expressed as mean ± standard deviation (SD) of 3 to 4 independent experiments.

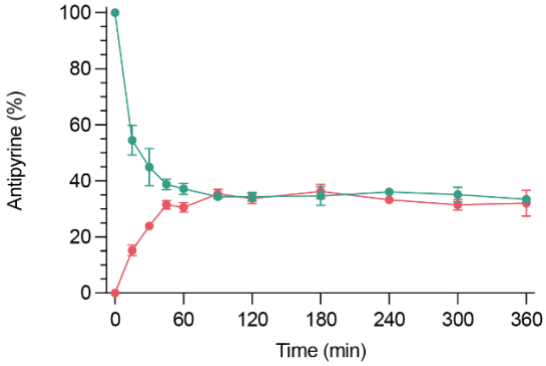


Figure 3S. Perfusion profile of antipyrine from control perfusions. Concentrations are expressed as a percentage (%) of initial analyzed concentration in the maternal sample (● maternal and ● fetal). All values are expressed as mean ± standard deviation (SD) of at least 3 independent experiments (except timepoint 360 min in which only 2 values are included).

Table 3S. Characteristics of placentae used, and data from individual perfusions with all study compounds (antipyrine, n=3; protopine, n=4; citalopram, n=4; and diazepam, n=5).

	Antipyrine			Protopine				Citalopram				Diazepam				
Experiment number	1	2	3	1	1	1	1	1	2	3	4	1	2	3	4	5
Placenta weight (g)	690	680	580	716	555	555	555	660	720	720	480	520	500	580	480	690
Cotyledon weight (g)	30.95	23.26	6.15	17.13	22.27	22.27	22.27	24.56	45.93	12.50	12.95	12.92	7.32	18.17	15.8	17.18
Volume loss* (M, mL)	-4	6	11	-7	4	4	4	1	-5	-11	14	-6	-2	0	0	-1
Volume loss* (F, mL)	8	10	4	6	3	3	3	5	8	4	9	4	4	0	11	3
Preparation time° (min)	42	14	29	17	22	22	22	25	18	13	30	15	15	15	15	18
Cannulation time§ (min)	16	15	30	30	13	13	13	35	40	19	19	27	21	18	22	23
Open pre-phase (min)	20	20	20	20	20	20	20	22	20	20	20	21	20	20	22	13
Close pre-phase (min)	20	20	20	23.5	21	21	21	22.5	20	20	20	25	24	26	24	43
Perfusion time (min)	300	360	360	240	360	360	360	360	360	300	360	360	360	360	360	360

* Total volume loss at the end of the perfusion time; M = maternal; F = fetal; ° time from birth to begin of cannulation; § time from cannulation to begin of open pre-phase.

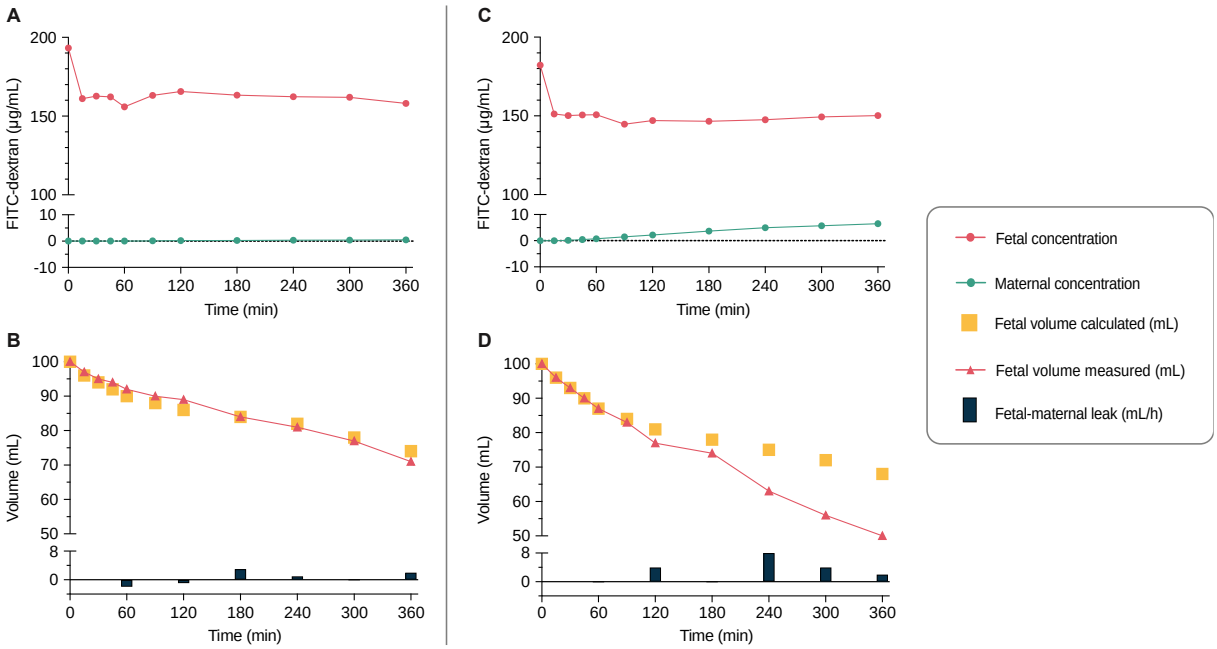


Figure 4S. Establishment and validation of the human *ex vivo* placental perfusion model. Assessment of the suitability of fluorescein isothiocyanate (FITC)-dextran (40 kDa) as a fetal capillary integrity marker when added to the fetal circuit at a concentration of 200 µg/mL. **(A)** A representative perfusion profile without a transfer of fluorescence (FITC-dextran) from the fetal to the maternal side occurs when the following two conditions were met: **(B)** the measured fetal volume corresponded to the calculated volume and a fetal-maternal (FM) leak of 4 mL/h was not exceeded. **(C)** A representative profile with a FITC-dextran transfer into the maternal circuit, if these conditions were violated: (**D**) the course of measured fetal volume in the corresponding perfusion deviated from the calculated volume, indicative of FM leaks (e.g. 8 mL/h at 240 min). A tracking of fetal and maternal volumes was therefore considered an adequate indicator for FM leaks, and no FITC-dextran was added in the subsequent placental perfusions.

Table 4S. U(H)PLC gradients, flow rate, and internal standards for the analysis of antipyrine, protopine, verapamil, diazepam, and citalopram. The mobile phase consisted of A (water with 5% MeCN and 0.1% formic acid) and B (MeCN with 0.1% formic acid). In the case of diazepam the mobile phase A contained only water and 0.1% formic acid.

Antipyrine				
	Time (min)	**%A**	**%B**	**Flow rate (mL/min)**	**Internal standard**
	0.00	100.0	0.0		
	0.50	100.0	0.0		
	3.00	37.89	62.11		
	3.01	0.0	100.0	0.4	Antipyrine-d3
	4.00	0.0	100.0		
	4.01	100.0	0.0		
	5.00	100.0	0.0		
Protopine				
	Time (min)	**%A**	**%B**	**Flow rate (mL/min)**	**Internal standard**
	0.00	100.0	0.0		
	1.00	100.0	0.0		
	3.00	0.0	100.0	0.5	Verapamil
	4.00	0.0	100.0		
	4.01	100.0	0.0		
	5.00	100.0	0.0		
Diazepam				
	Time (min)	**%A**	**%B**	**Flow rate (mL/min)**	**Internal standard**
	0.00	98.0	2.0		
	0.50	98.0	2.0		
	4.00	10.0	90.0		
	4.50	0.0	100.0	0.4	Diazepam-d5
	5.00	0.0	100.0		
	5.01	98.0	2.0		
	6.00	98.0	2.0		
Citalopram				
	Time (min)	**%A**	**%B**	**Flow rate (mL/min)**	**Internal standard**
	0.00	100.0	0.0		
	1.00	100.0	0.0		
	3.00	0.0	100.0	0.4	Citalopram-d4
	4.00	0.0	100.0		
	4.10	100.0	0.0		
	5.00	100.0	0.0		

Table 5S. MRM transitions and internal standards for the analysis of antipyrine, protopine, verapamil, diazepam, and citalopram. Gradients as in Table 4S.

Compound	Range (ng/mL)	MRM transitions	Internal standard	Ionization
Antipyrine	5 - 500	189.1 >104.0 189.1 > 56.1	Antipyrine-d3	ESI+
Protopine	5 - 250	354.14 > 189.1 354.14 > 149.0	Verapamil*	ESI+
Diazepam	10 - 1000	285.08 > 193.1 285.08 > 154.0	Diazepam-d5	ESI+
Citalopram	10 - 1000	324.9 > 109.1 324.9 > 262.1	Citalopram-d4	ESI+

* MRM transition 455.29 > 77.1.

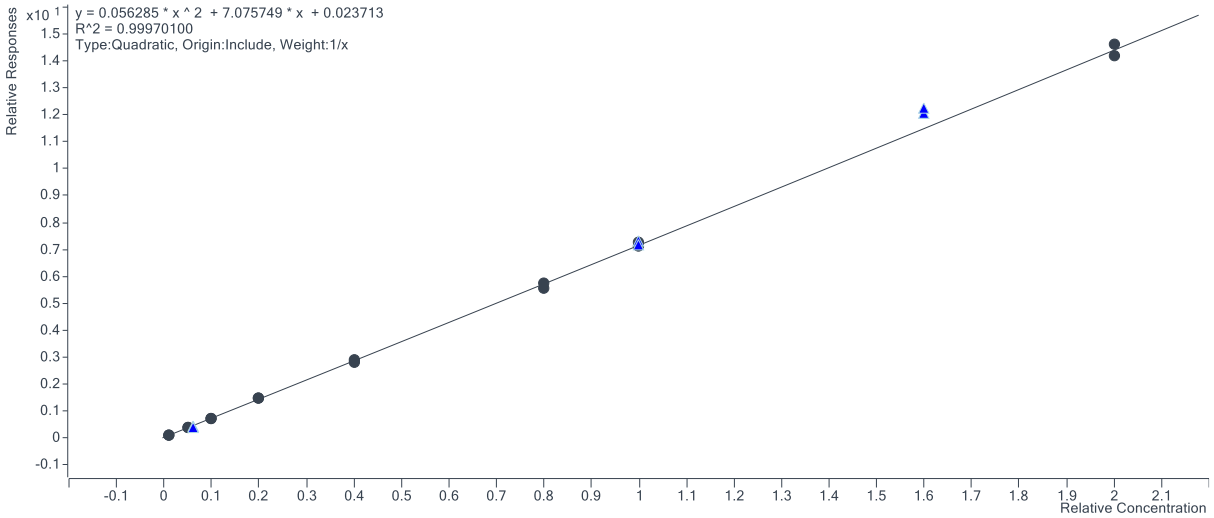


Figure 5S. Calibration curve of antipyrine (calibrators are shown as dots and quality controls [QCs] are shown as triangles).

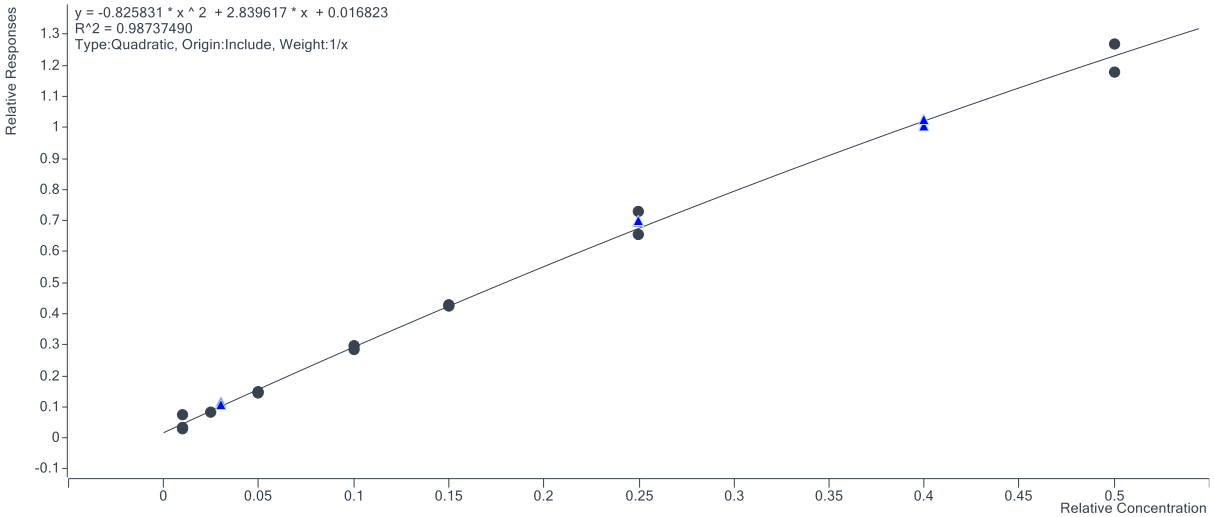


Figure 6S. Calibration curve of protopine (calibrators are shown as dots and quality controls [QCs] are shown as triangles).

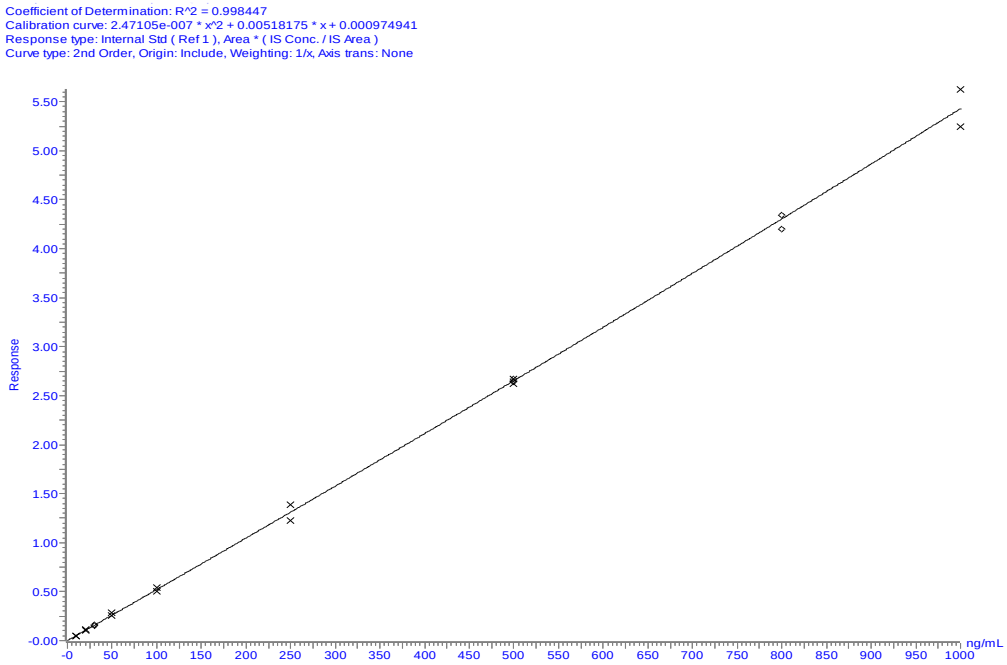


Figure 7S. Calibration curve of citalopram (calibrators are shown as x and quality controls [QCs] are shown as diamond).

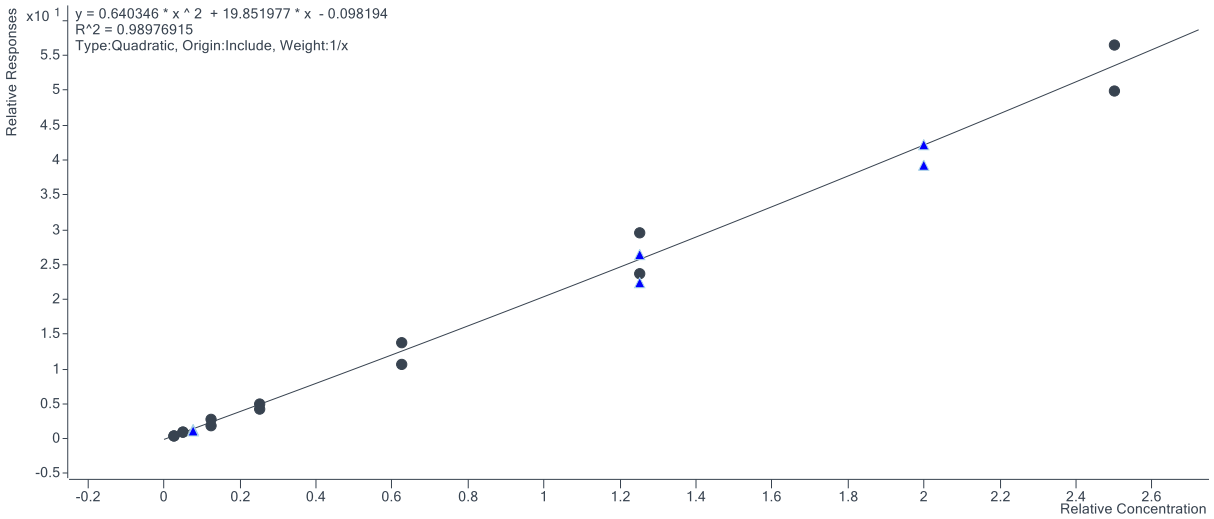


Figure 8S. Calibration curve of diazepam (calibrators are shown as dots and quality controls [QCs] are shown as triangles).

Table 6S. Calibrators and calibration curve parameters for the determination of antipyrine.
Response: $A \times Conc^2 + B \times Conc + C$, 1/X weighting, Quadratic regression, included origin (n=40).

	Concentration (ng/mL)							Regression parameters			
	5	**25**	**50**	**100**	**200**	**400**	**500**	**A**	**B**	**C**	**R²**
Mean	5.019	25.361	50.383	99.622	198.024	394.796	509.635	-8.02E-02	2.270	0.008	0.998
SD	0.398	1.386	2.803	2.936	6.501	12.441	17.382	1.75E-01			
CV%	7.937	5.467	5.563	2.947	3.283	3.151	3.151				
RE%	0.380	1.445	0.767	-0.378	-0.988	-1.301	1.927				

Table 7S. Calibrators and calibration curve parameters for the determination of protopine.
Response: $A \times Conc^2 + B \times Conc + C$, 1/X weighting, Quadratic regression, included origin (n=12).

	Concentration (ng/mL)							Regression parameters			
	5	**12.5**	**25**	**50**	**75**	**125**	**250**	**A**	**B**	**C**	**R²**
Mean	4.900	12.600	24.800	51.700	76.200	120.300	253.000	-1.98E-00	3.760	-0.007	0.996
SD	0.295	0.765	2.058	3.856	4.854	6.182	15.707	2.39E-00			
CV%	6.030	6.070	8.300	7.450	6.400	5.140	6.210				
RE%	-2.280	0.785	-0.980	3.400	1.579	-3.740	1.194				

Table 8S. Calibrators and calibration curve parameters for the determination of citalopram.
Response: $A \times Conc^2 + B \times Conc + C$, 1/X weighting, Quadratic regression, included origin (n=12).

	Concentration (ng/mL)							Regression parameters			
	10	**20**	**50**	**100**	**250**	**500**	**1000**	**A**	**B**	**C**	**R²**
Mean	10.300	19.400	51.000	102.200	253.100	490.600	1001.500	-1.43E-06	0.020	0.043	0.997
SD	0.815	1.207	3.875	6.408	12.177	31.771	52.529	1.31E-06			
CV%	7.900	6.220	7.600	6.270	4.800	6.480	5.250				
RE%	3.180	-2.955	1.980	2.180	1.250	-1.870	0.146				

Table 9S. Calibrators and calibration curve parameters for the determination of diazepam.
Response: $A \times Conc^2 + B \times Conc + C$, 1/X weighting, Quadratic regression, included origin (n=12).

	Concentration (ng/mL)							Regression parameters			
	10	**20**	**50**	**100**	**250**	**500**	**1000**	**A**	**B**	**C**	**R²**
Mean	10.300	19.600	49.000	101.700	251.600	498.500	986.200	3.83E-02	0.761	-0.021	0.994
SD	0.910	1.386	4.380	6.962	20.408	41.574	58.331	9.39E-02			
CV%	8.800	7.050	8.900	6.840	8.100	8.340	5.910				
RE%	3.330	-1.794	-1.940	1.730	0.639	-0.290	-1.380				

Table 10S. Quality control samples of antipyrine (n=40).

	QCL 15	QCM 250	QCH 400
Mean	16.100	239.400	380.500
SD	1.680	50.420	76.350
CV%	10.400	21.070	20.070
RE%	7.600	-4.250	-4.865

Table 11S. Quality control samples of protopine (n=12).

	QCL 15	QCM 125	QCH 200
Mean	16.900	125.900	202.800
SD	0.260	12.360	23.420
CV%	1.500	9.820	11.550
RE%	12.990	0.690	1.385

Table 12S. Quality control samples of citalopram (n=12).

	QCL 30	QCM 500	QCH 800
Mean	33.025	520.560	835.480
SD	0.860	16.730	50.480
CV%	2.700	3.240	6.030
RE%	8.670	3.360	4.663

Table 13S. Quality control samples of diazepam (n=12).

	QCL 30	QCM 500	QCH 800
Mean	30.600	504.400	801.200
SD	2.760	27.350	36.840
CV%	9.000	5.420	4.600
RE%	1.900	0.890	0.147

Table 14S. Carry-over assessment for antipyrine.

Run	Replicate	Peak area response (cts)				Individual carry-over (%)		Mean carry-over (%)	
		Blank sample		LLOQ					
		Analyte	IS	Analyte	IS	Analyte	IS	Analyte	IS
1	1	0.00	0.00	15483.14	222133.29	0.00	0.00	4.00	0.04
	2	1054.29	141.84	13171.49	183540.99	8.00	0.08		
2	1	900.18	0.00	11057.83	158834.00	8.14	0.00	7.69	0.04
	2	824.46	121.97	11378.04	160274.60	7.25	0.08		
3	1	776.10	126.21	6320.00	137976.60	12.28	0.09	8.86	0.17
	2	387.52	366.51	7125.06	152498.84	5.44	0.24		
4	1	108.88	292.98	8766.76	171126.49	1.24	0.17	1.58	0.18
	2	169.18	345.30	8826.13	180080.12	1.92	0.19		
5	1	0.00	0.00	10196.77	199575.63	0.00	0.00	2.21	0.00
	2	389.88	0.00	8809.64	216615.27	4.43	0.00		
6	1	2631.78	336.44	17131.43	143976.90	15.36	0.23	16.24	0.53
	2	3260.05	1623.08	19055.93	195630.14	17.11	0.83		
7	1	*5588.23	355.29	12784.71	124385.47	-	0.29	6.31	0.16
	2	998.41	50.59	15824.74	155791.82	6.31	0.03		
8	1	634.72	1613.31	12325.89	131973.29	5.15	1.22	5.20	1.17
	2	712.30	1524.55	13553.97	136836.17	5.26	1.11		
9	1	414.42	19.14	8142.70	75724.15	5.09	0.03	8.58	0.03
	2	1862.45	61.24	15422.24	149839.59	12.08	0.04		
10	1	1071.57	68.63	10048.99	105887.43	10.66	0.06	9.79	0.05
	2	1011.62	53.25	11353.70	120649.40	8.91	0.04		
11	1	2564.87	71.50	16715.36	142553.79	15.34	0.05	15.73	0.05
	2	2916.05	80.08	18096.49	149729.07	16.11	0.05		
12	1	1020.98	85.83	14592.02	140330.04	7.00	0.06	8.20	0.05
	2	1336.30	63.12	14218.52	142716.85	9.40	0.04		
13	1	837.07	66.31	12497.91	131122.86	6.70	0.05	8.49	0.05
	2	2948.15	65.65	28692.43	167114.61	10.28	0.04		
14	1	1232.57	284.99	16362.89	149697.19	7.53	0.19	7.53	0.19
	2	*10334.61	*89.71	18322.91	166673.99	-	-		
15	1	2132.27	99.25	12386.00	112776.64	17.22	0.09	14.56	0.08
	2	1528.94	87.59	12836.22	108165.64	11.91	0.08		
16	1	0.00	0.00	17787.36	172245.67	0.00	0.00	4.84	0.00
	2	877.25	0.00	9056.74	90599.25	9.69	0.00		
17	1	177.39	114.45	1912.78	39997.00	9.27	0.29	8.00	0.19
	2	165.10	46.87	2458.12	52289.93	6.72	0.09		
18	1	113.69	41.44	1859.26	39040.54	6.11	0.11	7.46	0.12
	2	177.74	58.88	2020.34	42213.96	8.80	0.14		
19	1	415.72	151.04	2147.98	52787.39	19.35	0.29	16.71	0.23
	2	423.71	146.43	3012.98	84257.39	14.06	0.17		
20	1	334.18	12.76	3290.16	84747.64	10.16	0.02	9.90	0.02
	2	298.87	25.83	3097.33	86228.19	9.65	0.03		
*Cal 1 outside acceptance criteria, carry-over not calculated.							**Mean carry-over**	8.19	0.16

Table 15S. Carry-over assessment for protopine.

Run	Replicate	Peak area response (cts)				Individual carry-over (%)		Mean carry-over (%)	
		Blank sample		LLOQ					
		Analyte	IS	Analyte	IS	Analyte	IS	Analyte	IS
1	1	359.33	209.10	8285.35	97552.87	4.34	0.21	4.20	0.31
	2	326.79	461.11	8031.53	111516.56	4.07	0.41		
2	1	1213.96	117.56	4957.28	73555.43	24.49	0.16	22.73	0.15
	2	1138.38	110.52	5426.01	79165.82	20.98	0.14		
3	1	91.71	201.65	8069.87	123427.64	1.14	0.16	1.13	0.14
	2	83.68	138.95	7451.73	129270.72	1.12	0.11		
4	1	200.34	133.66	9992.82	168839.27	2.00	0.08	3.15	0.10
	2	583.21	268.32	13547.80	210399.04	4.30	0.13		
5	1	254.09	705.94	9336.76	165637.38	2.72	0.43	1.90	0.28
	2	133.64	261.65	12376.77	185863.41	1.08	0.14		
6	1	150.42	81.08	9258.74	173662.54	1.62	0.05	2.39	0.07
	2	316.08	178.34	10013.84	200257.87	3.16	0.09		
							Mean carry-over	5.919	0.176

Table 16S. Carry-over assessment for citalopram.

Run	Replicate	Peak area response (cts)				Individual carry-over (%)		Mean carry-over (%)	
		Blank sample		LLOQ					
		Analyte	IS	Analyte	IS	Analyte	IS	Analyte	IS
1	1	12143.52	130.52	334833.44	23864.26	3.63	0.55	2.72	0.31
	2	8979.30	13.29	495520.31	20404.75	1.81	0.07		
2	1	261.93	13.56	6005.38	22682.23	4.36	0.06	3.80	0.06
	2	194.69	13.45	6002.61	22931.61	3.24	0.06		
3	1	280.06	6.42	7185.11	19199.77	3.90	0.03	3.63	0.02
	2	273.09	1.74	8126.70	26066.70	3.36	0.01		
4	1	247.25	6.52	7132.82	20700.86	3.47	0.03	3.42	0.05
	2	248.09	14.25	7371.08	24053.68	3.37	0.06		
5	1	1358.55	230.73	3639.41	59973.48	37.33	0.38	32.77	0.35
	2	1156.00	191.78	4097.65	61940.79	28.21	0.31		
6	1	1028.20	174.39	3601.83	59565.51	28.55	0.29	26.42	0.28
	2	898.50	162.33	3698.60	60280.36	24.29	0.27		
							Mean carry-over	12.126	0.176

Table 17S. Carry-over assessment for diazepam.

Run	Replicate	Peak area response (cts)				Individual carry-over (%)		Mean carry-over (%)	
		Blank sample		LLOQ					
		Analyte	IS	Analyte	IS	Analyte	IS	Analyte	IS
1	1	699.34	27.05	21728.50	68480.63	3.22	0.04	2.93	0.08
	2	644.56	77.08	24476.12	67962.90	2.63	0.11		
2	1	1642.00	72.00	42957.00	168257.00	3.82	0.04	4.32	0.05
	2	1173.00	56.00	24311.00	95654.00	4.82	0.06		
3	1	894.09	34.42	25148.81	104117.53	3.56	0.03	9.10	0.06
	2	2419.19	46.12	16526.26	52667.72	14.64	0.09		
4	1	778.72	64.97	20966.67	209367.04	3.71	0.03	2.89	0.07
	2	356.70	167.37	17301.90	141594.28	2.06	0.12		
5	1	438.00	20.00	29823.00	103765.00	1.47	0.02	1.36	0.02
	2	335.00	9.00	26665.00	79608.00	1.26	0.01		
6	1	137.00	5.00	25805.00	68408.00	0.53	0.01	0.93	0.01
	2	671.00	18.00	50813.00	109412.00	1.32	0.02		
							Mean carry-over	3.587	0.048

Chapter 5

Chapter 5

Placental passage of hyperforin, hypericin, and valerenic acid

Deborah Spiess[*], Vanessa Fabienne Abegg[*], Antoine Chauveau, Joshua Rath, Andrea Treyer, Michael Reinehr, Sabrina Kuoni, Mouhssin Oufir, Olivier Potterat, Matthias Hamburger, Ana Paula Simões-Wüst

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Safe medications for mild mental diseases in pregnancy are needed. Phytomedicines fromSt. John's wort and valerian are valid candidates, but safety data in pregnancy are lacking. The transplacental transport of hyperforin and hypericin (from St. John's wort), and valerenic acid (from valerian) was evaluated using the ex vivo cotyledon perfusion model (4 h perfusions, term placentae) and, in part, the in vitro Transwell assay with BeWo b30 cells. Antipyrine was used for comparison in both models. U(H)PLC-MS/MS bioanalytical methods were developed to quantify the compounds. Perfusion data obtained with term placentae showed that only minor amounts of hyperforin passed into the fetal circuit, while hypericin did not cross the placental barrier and valerenic acid equilibrated between the maternal and fetal compartments. None of the investigated compounds affected metabolic, functional, and histopathological parameters of the placenta during the perfusion experiments. Data from the Transwell model suggested that valerenic acid does not cross the placental cell layer. Taken together, our data suggest that throughout the pregnancy the potential fetal exposure to hypericin and hyperforin – but not to valerenic acid – is likely to be minimal.

Contributions of Deborah Spiess to this publication: performing placental perfusion experiments, analysis and interpretation of data, data visualization, writing major parts of the manuscript, and manuscript revision

[*] *These authors contributed equally to the work and should be considered as joint first authors*

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EDITED BY
Giada Crescioli,
University of Florence, Italy

REVIEWED BY
Kathleen Job,
The University of Utah, United States
Lukas Cerveny,
Charles University, Czechia

*CORRESPONDENCE
Matthias Hamburger,
matthias.hamburger@unibas.ch
Ana Paula Simões-Wüst,
anapaula.simoes-wuest@usz.ch

†Present address: Mouhssin Oufir, Oncodesign SA, Villebon-sur-Yvette, France

‡These authors have contributed equally to this work

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Transplacental passage of hyperforin, hypericin, and valerenic acid

Deborah Spiess[1,2‡], Vanessa Fabienne Abegg[2‡], Antoine Chauveau[2], Joshua Rath[1], Andrea Treyer[2], Michael Reinehr[3], Sabrina Kuoni[1], Mouhssin Oufir[2†], Olivier Potterat[2], Matthias Hamburger[2*] and Ana Paula Simões-Wüst[1*]

[1]Department of Obstetrics, University Hospital Zurich, University of Zurich, Zurich, Switzerland, [2]Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland, [3]Department of Pathology and Molecular Pathology, University Hospital Zurich, Zurich, Switzerland

Safe medications for mild mental diseases in pregnancy are needed. Phytomedicines from St. John's wort and valerian are valid candidates, but safety data in pregnancy are lacking. The transplacental transport of hyperforin and hypericin (from St. John's wort), and valerenic acid (from valerian) was evaluated using the *ex vivo* cotyledon perfusion model (4 h perfusions, term placentae) and, in part, the *in vitro* Transwell assay with BeWo b30 cells. Antipyrine was used for comparison in both models. U(H)PLC-MS/MS bioanalytical methods were developed to quantify the compounds. Perfusion data obtained with term placentae showed that only minor amounts of hyperforin passed into the fetal circuit, while hypericin did not cross the placental barrier and valerenic acid equilibrated between the maternal and fetal compartments. None of the investigated compounds affected metabolic, functional, and histopathological parameters of the placenta during the perfusion experiments. Data from the Transwell model suggested that valerenic acid does not cross the placental cell layer. Taken together, our data suggest that throughout the pregnancy the potential fetal exposure to hypericin and hyperforin – but not to valerenic acid – is likely to be minimal.

KEYWORDS

hyperforin, hypericin, valerenic acid, *Hypericum perforatum*, *Valeriana officinalis*, placental perfusion, pregnancy, U(H)PLC-MS/MS

Introduction

Pregnancy is a vulnerable period for mental disorders and/or symptoms. Studies from various countries (e.g., low-middle-income countries, United States, Sweden), reported a prevalence of 14%–20% for psychiatric diseases in pregnancy (Andersson et al., 2003; Marcus et al., 2003; Fisher et al., 2012). In Switzerland, the annual rate of

Abbreviations: BSA, bovine serum albumin; C_{cl}, electrical capacitance; DMSO, dimethyl sulfoxide; 96-DWP, 96-deepwell plate; EMA, European Medicine Agency; ESI, electrospray ionization; FM, fetal-maternal; FM ratio, fetal-maternal concentration ratio; HMPC, Committee on Herbal Medicinal Products; β-hCG, beta-human chorionic gonadotropin; IS, internal standard; MeCN, acetonitrile; MRM, multiple reaction monitoring; MW, molecular weight; NaF, sodium fluorescein; P_{app}, apparent permeability coefficient; PM, perfusion medium; SSRIs, selective serotonin reuptake inhibitors; TER, transepithelial electrical resistance.

perinatal women using mental health services accounted for 16.7% (Berger et al., 2017). Moreover, a Swiss cross-sectional survey revealed that more than 52.0% of the participants suffered from mental disorders and/or symptoms during pregnancy but only a few (1.6%) took synthetic psychoactive medications (Gantner et al., 2021). Mental disorders like mild depression, sleep disorders, and anxiety can lead to complications like preterm birth if left untreated (Dunkel Schetter, 2011). However, most synthetic drugs may not only cause side effects in the mother, but also cross the placental barrier and reach the fetus. Concerns on tolerability, teratogenicity and impact on neonatal outcomes exist and are, in part, supported by various studies (Sivojelezova et al., 2005; Rahimi et al., 2006; Grigoriadis et al., 2014; Yonkers et al., 2014; Gao et al., 2018). Pregnant women in need of medications such as selective serotonin reuptake inhibitors (SSRIs) and benzodiazepines therefore face the dilemma of either using or refraining from using them.

Phytomedicines are popular alternatives to synthetic medications. Many pregnant women use herbal medicines, in addition to or rather than synthetic drugs, probably as they perceive those alternatives to be the safer choice for their unborn child (Sarecka-Hujar and Szulc-Musiol, 2022). A recent Swiss survey revealed that 89.9% of pregnant women are using some type of herbal medicine (Gantner et al., 2021). Some healthcare professionals also tend to recommend phytomedicines and, hence, contribute to the trust in these products (Stewart et al., 2014). Most phytomedicines are available without prescription. In general, safety data for use during pregnancy are lacking for phytomedicines (Bernstein et al., 2021; Morehead and McInnis, 2021). For example, it is not known whether pharmacologically active compounds in these products can cross the placental barrier. Given the lack of data, the agencies responsible for approval of drugs require a warning label in the patient information of these products.

In the treatment of mild to moderate depression, St. John's wort (*Hypericum perforatum* L., Hypericaceae) is an alternative to SSRIs, as the clinical efficacy has been documented in several clinical trials (Linde et al., 2008). Hyperforin and hypericin are two characteristic compounds in St. John's wort. They have been shown to possess various CNS-related pharmacological activities but are not solely responsible for the antidepressant properties. As for most other phytomedicines, the entire extract has to be considered as the active ingredient (Butterweck and Schmidt, 2007; Nicolussi et al., 2020). A recent study based on data from Germany found that pregnant women mainly used St. John's wort in the first trimester, but simultaneous dispensation of other drugs that favour interactions and the observation of a relatively high rate of non-live births call for a thorough further safety investigation (Schäfer et al., 2021). Valerian *(Valeriana officinalis* L., Caprifoliaceae) is known for its sleep promoting and anxiolytic properties (Hattesohl et al., 2008). Valerenic acid, a characteristic compound in valerian, is an allosteric modulator of $GABA_A$ receptors (Becker et al., 2014), but again is not solely responsible for the clinical efficacy of phytomedicines containing valerian extracts. Valerian is favoured by many expecting mothers, as shown in a multinational study where valerian was among the five most frequently used herbal medicines (Kennedy et al., 2013). St. John's wort and valerian have been used for decades in Europe and have been labelled with "well established use" and "traditional use", respectively, by the Committee on Herbal Medicinal Products (HMPC) of the European Medicines Agency (EMA). However, they recommend neither of the herbs to be used during pregnancy due to insufficient toxicological data (European Medicines Agency, 2015; European Medicines Agency, 2021).

Relevant toxicological aspects in the context of pregnancy include effects on placental function and on transplacental transfer. The human placenta develops during pregnancy, at each gestational stage supplying the developing fetus with blood, nutrients, and oxygen, while also regulating the removal of waste products and carbon dioxide. In addition, it metabolises substances and releases hormones that influence the course of pregnancy, fetal metabolism and growth, and labour itself (Gude et al., 2004). The placenta also protects the fetus from infections, maternal diseases, and some xenobiotics including drugs. Most drugs pass through the placenta *via* passive or simple diffusion that is influenced by factors such as molecular weight (MW), degree of ionization, lipid solubility, protein binding, concentration gradient of the drug across the placenta, placental surface area/thickness, pH of maternal and fetal blood, and placental metabolism (van der Aa et al., 1998; Syme et al., 2004; Tetro et al., 2018). Other processes of drug transfer across the placenta include facilitated diffusion, active transport, and endocytosis. Once formed, the placental syncytiotrophoblast is the rate-limiting barrier separating the maternal and fetal circulation, with various transporters and enzymes located at the apical and basolateral membranes (Desforges and Sibley, 2010; Al-Enazy et al., 2017). Prior to their formation, a monolayer of precursor cells (cytotrophoblasts) held together by tight junctions exerts the barrier function (Prouillac and Lecoeur, 2010). Data on transplacental passage of drugs can be obtained using the *ex vivo* human placental perfusion model (with term placentae) representative of the late stage of pregnancy (Myllynen and Vahakangas, 2013). It is considered to be the gold-standard among placental transfer models (Panigel et al., 1967; Malek et al., 2009; Grafmuller et al., 2013), and we have recently shown its usefulness for studying the transplacental transfer of phytochemicals (Spiess et al., 2022a). *In vitro* Transwell models utilising monolayers of confluent, human, non-differentiated placental cells, on the other hand, reflect the transfer through a continuous layer of cytotrophoblasts (Bode et al., 2006; Vähäkangas and Myllynen, 2006; Prouillac and Lecoeur, 2010).

In the present study, we investigated the transplacental passage of hyperforin, hypericin, and valerenic acid (Figure 1) using the *ex vivo* term placental perfusion model. In this model, we also investigated their effects on metabolic, functional, and histopathological properties of placental tissue. In case of valerenic acid, an *in vitro* Transwell model based on the human placental BeWo b30 cell line (Li et al., 2013) was used in addition.

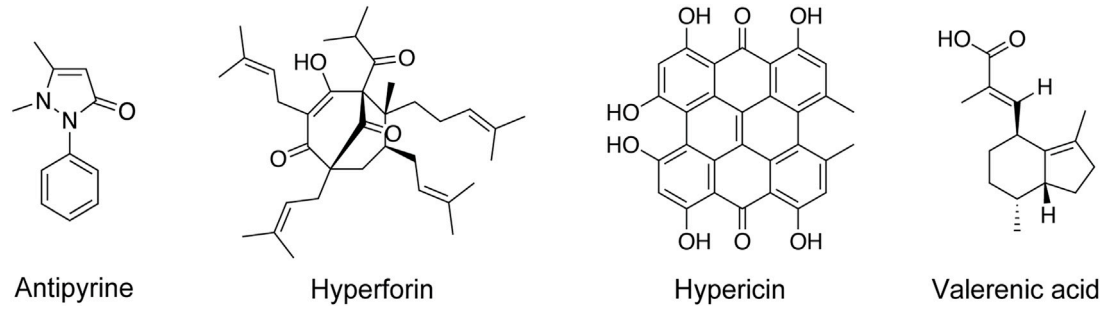

FIGURE 1
Chemical structures of investigated phytochemicals and antipyrine.

Materials and methods

Chemicals, reagents, and study compounds

All solvents were of UPLC grade. Acetonitrile (MeCN) was purchased from Merck. Methanol was from Avantor Performance Materials Poland. Purified water was obtained from a Milli-Q integral water purification system. Dimethyl sulfoxide (DMSO) was supplied by Scharlau, and formic acid was from BioSolve. Antipyrine, hyperforin dicyclohexylammonium salt and bovine serum albumin (BSA) were obtained from Sigma-Aldrich, and antipyrine-d_3 from HPC Standards. Valerenic acid was purchased from Extrasynthese and PhytoLab, and hypericin was from Carbosynth. Warfarin was purchased from Toronto Research Chemicals, and digoxin from Sigma-Aldrich.

Ex vivo human placental perfusion

Placentae collection

Placentae were collected with informed written consent from women with uncomplicated term pregnancies immediately after undergoing primary caesarean section. This procedure was approved by the Ethics Committee of the Canton of Zurich (KEK-StV73 Nr. 07/07; 21 March 2007). All research was performed in accordance with the Declaration of Helsinki and other relevant guidelines/regulations. All placentae were verified to be negative for HIV, HBV, SARS-CoV-2, and twin pregnancy donors were not included in the study. Placentae with a ragged maternal surface (visible disruptions; macroscopic tissue trauma), evidence of basal plate fibrin deposition, suspected placental infarction or too little fetal membrane (on the disk of the placenta) were not considered for perfusion. Supplementary Table S2 gives an overview of the experimental conditions and characteristics of all placentae used: a total of 11 placentae (donated by 11 women) were suitable for perfusion, 3-4 individual placentae were used for each test substance.

Equipment and experimental procedure of perfusion

The *ex vivo* human placental perfusion model was adapted from the models of Schneider (Schneider, 1972) and Grafmüller (Grafmuller et al., 2013), and has been described in detail (Spiess et al., 2022a). In brief, one villous tree of a cotyledon (placenta lobule) was perfused by cannulation of the chorionic artery and corresponding vein. Three blunt cannulae were inserted into the intervillous space to reconstruct the maternal circuit and to allow the transplacental exchange through the perfusion medium (PM). The PM consisted of Earle's buffer (1 part), cell culture medium 199 (2 parts; Sigma-Aldrich), and supplements (BSA, dextran, glucose, sodium bicarbonate, amoxicillin, and heparin). The fetal perfusate was gassed with 95% N_2/5% CO_2, and the maternal perfusate with 95% air/5% CO_2 instead. Two heating magnetic stirrers ensured a constant distribution of study compounds in both reservoirs. A physiological temperature of 37°C was maintained with flow heaters (heating columns) and a water bath. Digitally controlled peristaltic pumps (Ismatec) transported the fetal and maternal perfusates at a rate of 3 and 12 mL/min, respectively. The perfusion experiment, including a 20 min non-recirculating (open) and a 20 min recirculating (closed) preparatory phase, started with equal volumes of 100 mL fresh PM. The study compound and antipyrine were added to the perfusate of the maternal circuit, both at a final concentration of 5 µM (≙ 941 ng/mL antipyrine, 2′684 ng/mL hyperforin, 2′522 ng/mL hypericin, and 1′172 ng/mL valerenic acid).

The dissolution and adherence of the compounds to the perfusion equipment and to the tubing system was assessed before starting perfusions with human placentae. All study compounds were pumped over a period of 240 min through an empty (i.e., in the absence of placental tissue) perfusion chamber comprising only the maternal circuit. The compounds were directly dissolved in PM at a final concentration of 5 µM (100 mL reservoir) and individually tested in three independent experiments (n = 3).

Sample preparation and quality controls

Antipyrine served as connectivity (positive) control in all placental perfusions to verify the overlap of the maternal and the fetal circulation. The stability of volumes in each reservoir ensured the integrity of the circuits and served for the detection of fetal-maternal (FM) leaks (≤4 mL/h). Throughout the perfusion additional quality control measures included a physiological pH-range (7.2 ± 0.1) and a controlled fetal perfusion pressure (≤ 70 mmHg). Samples were taken at defined timepoints over a 240 min period, immediately centrifuged, and stored in glass

micro-inserts (VWR) at −80°C for bioanalytical analysis. A blood gas analysis (pH, pO_2, pCO_2, glucose, lactate; ABL800 FLEX) of fetal and maternal samples was performed to ensure viability and metabolic activity of placental tissue during perfusion. The production of the placental hormones beta-human chorionic gonadotropin (β-hCG) and leptin was monitored by standard ELISA to assess tissue functionality *ex vivo* [see (Spiess et al., 2022a) and references therein]. For β-hCG, flat-bottom microplates were coated with rabbit polyclonal anti-hCG antibody (Agilent Dako) at a 1:1′000 dilution and the mouse monoclonal anti-hCG (abcam; 1:5′000) served as secondary antibody. The peptide hormone hCG (Lucerna-Chem) was used as reference standard; standard final concentrations of between 100 mU/mL and 2.5 mU/mL were prepared by serial dilution in seven steps and used in each plate. The goat anti-mouse-IgG-horseradish peroxidase conjugate (abcam) antibody was used at a 1:5′000 dilution. The substrate consisted of O-phenylenediamine dihydrochloride. Intra-assay CV% was ≤10%, inter-assay CV% was <6% at 100 mU/mL and <15% at 10 mU/mL. For leptin, microtiter plates were coated with mouse monoclonal anti-human leptin/OB (R&D Systems; 1: 250). The second antibody was biotinylated monoclonal mouse anti-human leptin/OB (R&D Systems), used at a dilution of 1:1′000. The standard was recombinant human leptin (R&D Systems); standard final concentrations of between 2′000 pg/mL and 15.6 pg/mL were prepared by serial 1:2 dilution in seven steps and used in each plate. Intra-assay CV% was ≤10%, inter-assay CV% was <3% at 2′000 pg/mL and <15% at 250 pg/mL. The conjugate streptavidin-horseradish peroxidase (Southern Biotechnology Associates, 1:4′000) was added and the plate incubated for 60 min. For the development, ready-to-use tetramethylbenzidine substrate solution (Thermo Fischer) was used.

Histopathological evaluation

Immediately after perfusion the placentae were fixed in 4% paraformaldehyde for at least 24 h. Tissue samples from representative placental tissue sections, each from perfused, non-perfused, and transitional area were prepared to perform a pathological examination. For this purpose, the tissue was embedded in paraffin, cut (∅ 2-3 µm), and stained (standard hematoxylin-eosin stain, Braun-Brenn modified Gram stain) according to the standards of routine histopathological diagnosis of the Department of Pathology and Molecular Pathology (University Hospital of Zurich). The tissue of the non-perfused specimens was examined with regard to general placental pathologies as described in routine diagnostics (Turowski et al., 2012; Khong et al., 2016). The blood void and width of the intervillous space and fetal blood vessels in the chorionic villi provided information about the quality of perfusion, and particular attention was paid to the presence of intravascular thrombi. General signs of degeneration, such as vacuolization of the cytotrophoblast, villous vascular endothelium viability, and formation of hydropic villous changes compared with non-perfused tissue indicated whether tissue damage might have occurred during perfusion. Microscopic effects and placental tissue damage in the perfused area were expressed in relative amounts (%) to the non-perfused tissue.

In vitro permeability assay

Cell culture

BeWo b30 choriocarcinoma cells were obtained from Dr. Tina Buerki-Thurnherr (Empa, St. Gallen, Switzerland), with permission from Dr. Alan L. Schwartz (Washington University School of Medicine, St. Louis MO, USA), and were cultivated in F-12 K Nut Mix supplemented with 10% heat inactivated FBS, antibiotics (100 U/mL penicillin, 100 µg/mL streptavidin), and 2 mM L-glutamine (all from Gibco). The cells were cultivated in a humidified incubator at 37°C and 5% CO_2 atmosphere.

Monolayer formation on cell culture inserts

BeWo b30 cells were cultured on Transwell® polycarbonate membrane inserts (24-well format; 0.4 µm pore size, 0.33 cm^2 cell growth area, 200 µL apical volume, 1′000 µL basolateral volume; Corning, Sigma-Aldrich) at a density of 60′000 cells/well. These inserts were then cultivated in a cellZscope (nanoAnalytics) at 37°C/ 5% CO_2. Cell culture medium was replaced every 2 days up to 11 days to find the best possible conditions.

Evaluation of monolayer formation

Measurement of transepithelial electrical resistance (TER) was used to assess the tightness of a cell-to-cell barrier, and the electrical capacitance C_{cl}, provided additional information about the properties of the cell layer (e.g., presence of microvilli and other membrane extrusions). TER and C_{cl} values were recorded in 15 min time intervals, using a cellZscope®. TER values were corrected for the surface area (Ωcm^2) and the reference resistance (well with the same filter insert and medium, but absence of cells). Moreover, a permeability assay with sodium fluorescein (NaF) was performed on days 7–11 (n = 3). The basolateral compartment of a transparent 24-well plate consisted of PBS only (1′000 µL) while NaF (5 µM in PBS; 200 µL) was added to the apical compartment for 60 min. The control consisted of cell-free inserts (n = 3). Basolateral samples (50 µL) were directly added to black Nunc MaxiSorp microtiter plates, and concentrations were determined using a Cytation 3 fluorescence microplate reader (BioTek Instruments; excitation wavelength 460 nm; emission wavelength 515 nm).

Immunocytochemical staining of cells on inserts

BeWo b30 cells were stained with fluorescent probes for nuclei and cytoplasm as follows. Cells were fixed in 4% paraformaldehyde (Artechemis) for 20–30 min. Afterwards they were permeabilized and blocked with 0.3% Triton-X-100 (Sigma) in 1% BSA (Thermo Scientific) in PBS (Gibco) for another 20–30 min at room temperature on a shaker (50 rpm). Then, cells were incubated at room temperature on a shaker (50 rpm) for 4–6 h, wrapped in tinfoil, with a 1:10′000 solution of 4′,6-diamidino-2-phenylindole (DAPI; Sigma) and a 1:400 phalloidin-rhodamine (Invitrogen) solution diluted with 0.1% Triton X-100 in 1% BSA/PBS. Cells were then extensively washed with PBS, and the insert membranes were embedded between glass cover slides using Mowiol 4–88 (Sigma-Aldrich) to obtain flat membranes. The images were acquired with a Leica CTR 6000 microscope (Leica Microsystems) and the corresponding Leica Application Software X.

TABLE 1 ESI-MS conditions for the analysis of test compounds.

Compound	Range (ng/mL)	MRM transitions	Internal standard	Ionization
Hyperforin	2.5–250	535.38 > 383.2	Warfarin	ESI-
		535.38 > 313.2		
Hypericin	10–1′000	502.8 > 405.1	Digoxin	ESI-
		502.8 > 433.1		
Valerenic acid	10–1′000	232.8 > 41	Digoxin	ESI-
		232.8 > 84		
Antipyrine	5–500	189.1 > 104	Antipyrine-d_3	ESI+
		189.1 > 56.1		

Permeability assay

BeWo b30 cells were cultured in transparent 24-well plates under the same conditions as mentioned above and transferred to the cellZscope® at day 8 to record TER and C_{cl} values. After 24 h (day 9) and when a TER of 30–60 Ωcm^2 was reached and C_{cl} was between 0.5 and 5.0 μF/cm^2, the permeability assay was initiated by adding valerenic acid (5 μM; in HBSS with 4% BSA) to the apical compartment, while the basolateral compartment contained 1′000 μL of HBSS only. In addition, antipyrine (5 μM) was added along with the test substance as a control. Samples (150 μL apical, 800 μL basolateral) were collected at each time point (0, 15, 30, 60 min; one insert per time point). After 60 min, a part of the inserts was transferred back to the cellZscope® to monitor TER and C_{cl} values during another 24 h. The cells grown on the other part of the inserts were quickly washed with 200 μL of cold HBSS and then lysed with 700 μL acetonitrile (100 μL apical, 600 μL basolateral) for 40 min on an orbital shaker (450 rpm, room temperature) to determine the test substance cell contents.

Calculation of permeability coefficients and recovery

Apparent permeability coefficients (P_{app}) were calculated according to the following Eq. 1:

$$P_{app}\ (cm/s) = \frac{\Delta Q/\Delta t}{AC_{A0}} \tag{1}$$

where ΔQ/Δt is the rate of amount transported to the receiver compartment, A is the membrane surface area (0.33 cm^2), and C_{A0} is the initial concentration in the apical compartment.

The clearance values were calculated according to the following Eq. 2 (Neuhaus et al., 2008):

$$Clearance\ (\mu L) = \frac{C_{Bn}V_B}{C_A - \left(\frac{V_B}{V_A} \cdot \sum C_{Bn-1}\right)} \tag{2}$$

where C_{Bn} and V_B are the concentration and volume in the basolateral compartment at a specific timepoint (n), respectively; C_A and V_A are the concentration and volume in the apical compartment, respectively, and C_{Bn-1} is the total amount of substance found in the basolateral compartment up to the previous timepoint (n-1).

Recovery (mass balance) of each compound was calculated according to the following Eq. 3:

$$Recovery\ (\%) = \frac{C_{Af}V_A + C_{Bf}V_B + C_{Cf}V_C}{C_{A0}V_A} \cdot 100 \tag{3}$$

where C_{Af}, C_{Bf} and C_{Cf} are the final compound concentrations in apical, basolateral, and cellular compartments, respectively; C_{A0} is the initial concentration in the apical compartment, and V_A, V_B and V_C are the volumes in the respective compartments.

LC-MS/MS analysis

Instrument and chromatographic conditions

U(H)PLC-MS/MS measurements were performed on a 6460 Triple Quadrupole MS system with a 1290 Infinity LC system equipped with a binary capillary pump G4220A, column oven G1316C, and multisampler G7167B (all Agilent). Quantitative analysis by MS/MS were performed with electrospray ionization (ESI) in MRM (multiple reaction monitoring) mode. Desolvation and nebulization gas was nitrogen. MS/MS data were analyzed with Agilent MassHunter Workstation software version B.07.00. The temperature of the autosampler was 10°C. An Acquity UPLC HSS T3 column (1.8 μm; 100 mm × 2.1 mm) (Waters) was used for separation of the analyte and the internal standard (IS), except for hyperforin and its IS warfarin where an Acquity UPLC CSH Phenyl-Hexyl column (1.7 μm; 50 mm × 2.1 mm) was used. The mobile phase consisted of purified water with 5% MeCN containing 0.1% formic acid (A1) and MeCN containing 0.1% formic acid (B1). Analyses of hypericin and valerenic acid including the IS digoxin were performed on an Acquity UPLC system containing a binary pump, autosampler, and column heater, connected to an Acquity TQD (all Waters). Desolvation and nebulization gas was nitrogen, and collision gas was argon. Flow rate for analysis of all compounds was 0.4 mL/min. The column used was an Acquity UPLC BEH C18 (1.7 μm; 50 mm × 2.1 mm) (Waters). The autosampler temperature was set at 10°C, and the column temperature at 55°C. The mobile phase consisted of purified water containing 0.1% NH_4OH at pH 10.7 (A2) and MeCN/purified water (9:1) containing 0.1% NH_4OH (B2). U(H)PLC

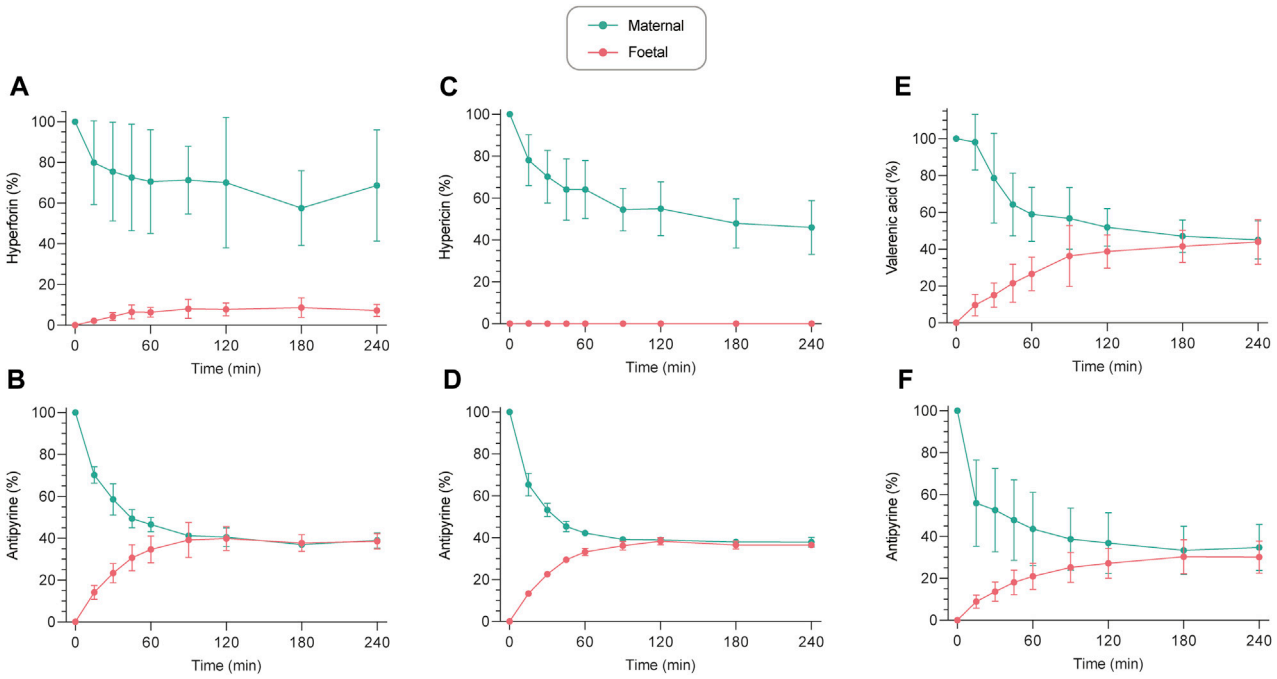

FIGURE 2
Ex vivo human placental perfusion profiles of hyperforin **(A)**, hypericin **(C)**, and valerenic acid **(E)** with corresponding connectivity controls (antipyrine) (**(B,D,F)**, respectively). Concentrations are expressed as a percentage (%) of the initially analyzed concentration in the maternal sample. All values are expressed as mean ± SD of three to four independent experiments. Perfusion profiles with absolute concentrations (ng/mL) can be found in Supplementary Figure S1.

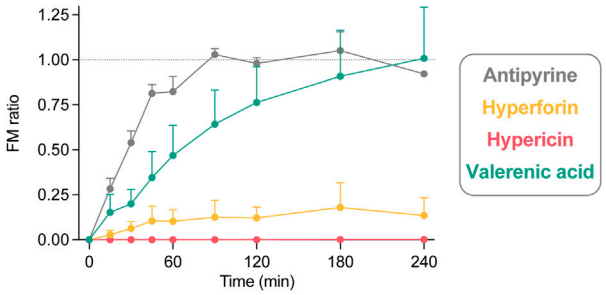

FIGURE 3
The fetal-maternal concentration ratio (FM ratio; fetal concentration divided by maternal concentration) calculated for each timepoint of hyperforin (n = 4), hypericin (n = 3), and valerenic acid (n = 4), in comparison with antipyrine (n = 3) from control perfusions. The FM ratio is 1.0 when the fetal and maternal concentrations are equal. All data are expressed as mean ± SD.

gradients used for analysis of antipyrine, hyperforin, hypericin, and valerenic acid are given in Supplementary Table S3 (Supplementary Material). MS/MS data were analyzed with MassLynx software version 4.1 (Agilent).

Standards and stock solutions

Stock solutions of analytes and IS (Table 1) were prepared as previously described (Spiess et al., 2022a). Details for the calibration curves can be found in Supplementary Figures S8–S11 (Supplementary Material) and in Supplementary Tables S4–S15 (Supplementary Material).

Sample extraction in placenta perfusion medium for antipyrine, hypericin, and valerenic acid

To 200 µL of analyte in PM were added 100 µL of the IS solution, 200 µL of 6% BSA in water, and 800 µL MeCN (1′000 µL for antipyrine). The samples were mixed for 10 min at room temperature on an Eppendorf Thermomixer (1′400 rpm), and finally centrifuged at 10°C for 20 min at 13′200 rpm/16′100 rcf (Centrifuge 5415 R, Eppendorf). An aliquot of 1′100 µL (1′300 µL for antipyrine) supernatant was collected and transferred into a 96-deepwell plate (96-DPW, Biotage) and dried under nitrogen gas flow (Evaporex EVX-96, Apricot Designs). Samples were redissolved with 200 µL of a mixture of A2 and B2 (65:35), followed by 45 min of shaking on an Eppendorf MixMate. Antipyrine was redissolved with 200 µL of A1 and B1 (65:35). Injection was done in full loop mode (2 µL) from the 96-DWP.

Sample extraction in placenta perfusion medium for hyperforin

150 µL of the IS in MeOH were added to 50 µL of sample. After centrifugation at 10°C for 20 min at 13′200 rpm/16′100 rcf (Centrifuge 5415R, Eppendorf), 50 µL of supernatant were collected and transferred into HPLC vials. Vials were centrifuged for additional 20 min prior to U(H)PLC-MS/MS analysis in full loop mode (2 µL).

TABLE 2 Predicted physicochemical properties of study compounds.

Compound	Molecular weight (g/mol)	pK_a	No. of hydrogen bond donors	clogD $_{pH\ 7.2}$	Physiological charge at pH 7.2		
					0 (% of total)	−1 (% of total)	−2 (% of total)
Hyperforin	536.8	4.5	1	7.6	0.2	99.8	0.0
Hypericin	504.4	6.9	6	7.3	30.7	59.3	9.9
Valerenic acid	234.3	4.9	1	2.3	0.5	99.5	0.0
Antipyrine	188.2	No acid p*Ka*	0	0.7	100.0	0.0	0.0

Sample extraction in placenta perfusion medium for Transwell samples

For valerenic acid, 150 µL of the IS in MeOH were added to 50 µL of sample, and the mixture centrifuged for 20 min at 13′200 rpm/16′100 rcf at 10°C (Centrifuge 5415R, Eppendorf). An aliquot (50 µL) of the supernatant was collected and transferred into HPLC vials. Vials were centrifuged for additional 20 min prior to U(H)PLC-MS/MS analysis in full loop mode (2 µL).

Stability assay in placental homogenate

The stability of hyperforin, hypericin, and valerenic acid was assessed in PBS, PM and placental homogenates (prepared according to (Spiess et al., 2022a)) over a period of 360 min, as previously published (Riccardi et al., 2020)). In short, after spiking the various matrices with the study compounds, samples were either processed immediately for U(H)PLC-MS/MS analysis (C0), or kept at 4°C/37°C on an orbital shaker (600 rpm) for 360 min before processing for U(H)PLC-MS/MS analysis. Samples were processed *via* solid phase extraction or protein precipitation prior to analysis.

Data processing and calculations

Concentrations in the placental perfusion profiles (Figure 2) and system adherence tests (Figure 4) were expressed as a percentage (%) of the maternal concentration at the beginning of the perfusion, whereby the maternal concentration, measured in the maternal reservoir, was adjusted to the total volume of the full maternal circuit (maternal reservoir and dead volume of the system). The FM concentration ratio (FM ratio; Figure 3) was calculated for each timepoint and plotted against the perfusion time (min). The final recovery (%) is the sum of the amounts of study compound present in both perfusates at the end of a perfusion, and the sample removed during the experiment. Glucose consumption and lactate production are presented as the sum of changes (from the beginning to the end of perfusion) in total content (µmol) in both circuits, normalized by total perfusion time (min) and weight (g) of perfused cotyledon. The net release rate of placental hormones – β-hCG (U) and leptin (ng) – during the placental perfusion was also normalized by total perfusion time (min) (see (Spiess et al., 2022b) for equations).

Statistical data analysis

Multiple group comparisons were performed for the glucose consumption, lactate production, β-hCG and leptin production using the non-parametric Mann–Whitney U with GraphPad Prism (version 9.3.1 for macOS; GraphPad Software). Probability values of $p \leq 0.05$ were considered statistically significant. Data are expressed as mean ± SD of three to four independent experiments.

Results

In silico predictions of physichochemical properties of the test substances

Hyperforin, hypericin and valerenic acid (Figure 1) exhibit rather differing physicochemical properties (Table 2), as determined by the softwares QikProp (Schrodinger LLC) and ACD/Percepta (ACD/Labs release 2020.1.1). Hyperforin and hypericin have both MWs that are markedly higher than that of valerenic acid (and the positive control antipyrine). Hyperforin and hypericin are predicted to be substantially more lipophilic than valerenic acid, which in turn is more lipophilic than the positive control antipyrine, as reflected by the $cLogD_{pH7.2}$ values (Table 2). Hypericin has a markedly higher number of hydrogen bond donors than all other compounds. Finally, the pK_a values of hyperforin and valerenic acid differ as well. At pH 7.2 of the perfusion experiments, hypericin is present in charge states between 0 and −2, while hyperforin and valerenic acid occur essentially in the single charge state of −1. In contrast, antipyrine is fully uncharged (Table 2).

Ex vivo characterisation of transplacental transfer

The transplacental transfer of hyperforin, hypericin and valerenic acid resulted in three distinctly different profiles (Figure 2). Hyperforin showed little transfer to the fetal circuit, despite a decrease in the maternal circuit. After 240 min of perfusion, only 7.2% of initial concentration appeared in the fetal compartment, while 68.6% of hyperforin remained on the maternal side (Figure 2A). Hypericin did not cross the human placental barrier within 240 min, while the concentration in the maternal compartment decreased to

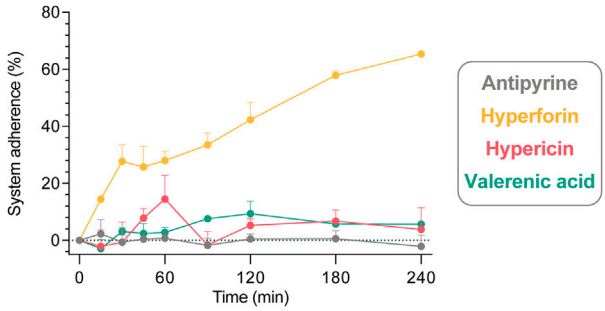

FIGURE 4
Adherence of study compounds in a 240 min system adherence test (circulation of study compounds through an empty perfusion chamber comprising only the maternal circuit). All compounds were tested individually in three independent experiments, and data are expressed as mean ± SD. Displayed is the percentage (%) of compound (of initially analyzed concentration in the maternal sample) that adheres to the equipment: antipyrine (−2.2% ± 7.0%), hyperforin (65.4% ± 0.7%), hypericin (3.8% ± 13.1%), and valerenic acid (5.6% ± 1.9%) at 240 min.

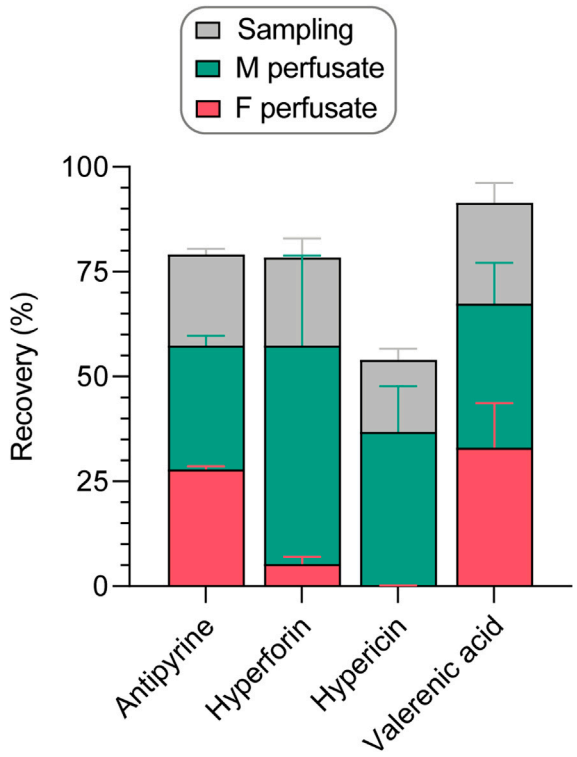

FIGURE 5
Recovery of study compounds in the human *ex vivo* placental perfusion system, expressed as percentage (%) of amount analyzed in the maternal sample at the beginning of the perfusion. The final recovery was calculated as the sum of compound present in fetal and maternal perfusates at the end of a perfusion, and the amounts sampled during the perfusion from fetal and maternal perfusates (Supplementary Table S1). All data are represented as mean ± SD of three to four independent experiments.

approximately 46% (Figure 2C). At the same time, antipyrine as a connectivity control reached an equilibrium after 120 min (Figures 2B, D). For valerenic acid, a gradual increase in the fetal compartment and a concomitant decrease in the maternal compartment was observed, reaching an equilibrium after 240 min (44.0% [fetal] vs. 45.0% [maternal] of initial concentration; Figure 2E). The integrity of maternal and fetal circuits was again confirmed with antipyrine (Figure 2F). Perfusion profiles with absolute concentrations can be found in Supplementary Figure S1.

The FM ratio (Figure 3) of hyperforin reached a maximum of 0.18 after 180 min, thereby confirming that only minor amounts crossed the placental barrier. In contrast, the FM ratio of hypericin was zero, as the compound could not be detected in the fetal circuit. For valerenic acid, the FM ratio was 1.01 after 240 min, reflecting the identical concentrations in the fetal and maternal compartments. Antipyrine as a positive control reached an equilibrium between fetal and maternal concentrations after 90 min (FM ratio of 1.03), which remained unchanged during the course of the experiment (FM ratios of 0.98, 1.05, 0.92 at 120, 180, and 240 min, respectively).

Recovery and stability of the test substances

The system adherence tests (empty perfusions; Figure 4) revealed that negligible proportions of hypericin, valerenic acid and antipyrine were lost over a period of 240 min (calculated values of 3.8%, 5.6%, and −2.2% of initial concentration, respectively). The relative amount of hyperforin which adhered to the perfusion equipment after 240 min was significantly higher (65.4%).

Apart from the system adherence test, several aspects must be considered for the recovery calculations of study compounds during placental perfusions (Figure 5). After 240 min of perfusion, the compounds were distributed in the two compartments (fetal vs. maternal) in the following proportions: antipyrine (27.8% vs. 29.5%), hyperforin (5.3% vs 52.2%), hypericin (0.1% vs. 36.7%), and valerenic acid (33.1% vs. 34.4%). As shown in Supplementary Table S1 and in Figure 5, 17.2%–24.1% of the test compounds were removed by sampling during the perfusion, corresponding to one-fourth of the final recovery. When assessing the final recovery in the placenta perfusions (without considering the results of the independent system adherence test), the following values were obtained: 79.2% ± 2.6% for antipyrine, 78.4% ± 23.0% for hyperforin, 54.0% ± 13.7% for hypericin, and 91.5% ± 19.1% for valerenic acid.

Limited stability of study compounds in the various matrices could lead to misleading results. Therefore, their stability was assessed over 360 min in three different matrices that were relevant for our experiments (PBS, PM, and three placental homogenates [Donors 1–3]) and at two temperatures (4°C, 37°C) (Figure 6). Hyperforin and hypericin were less stable over 360 min at 4°C and 37°C in PBS compared to PM, while the stability data of valerenic acid were very comparable in PBS and PM (approx. 100%). In addition, the solubility of hyperforin was higher in PM than in PBS (121% vs. 100%, Supplementary

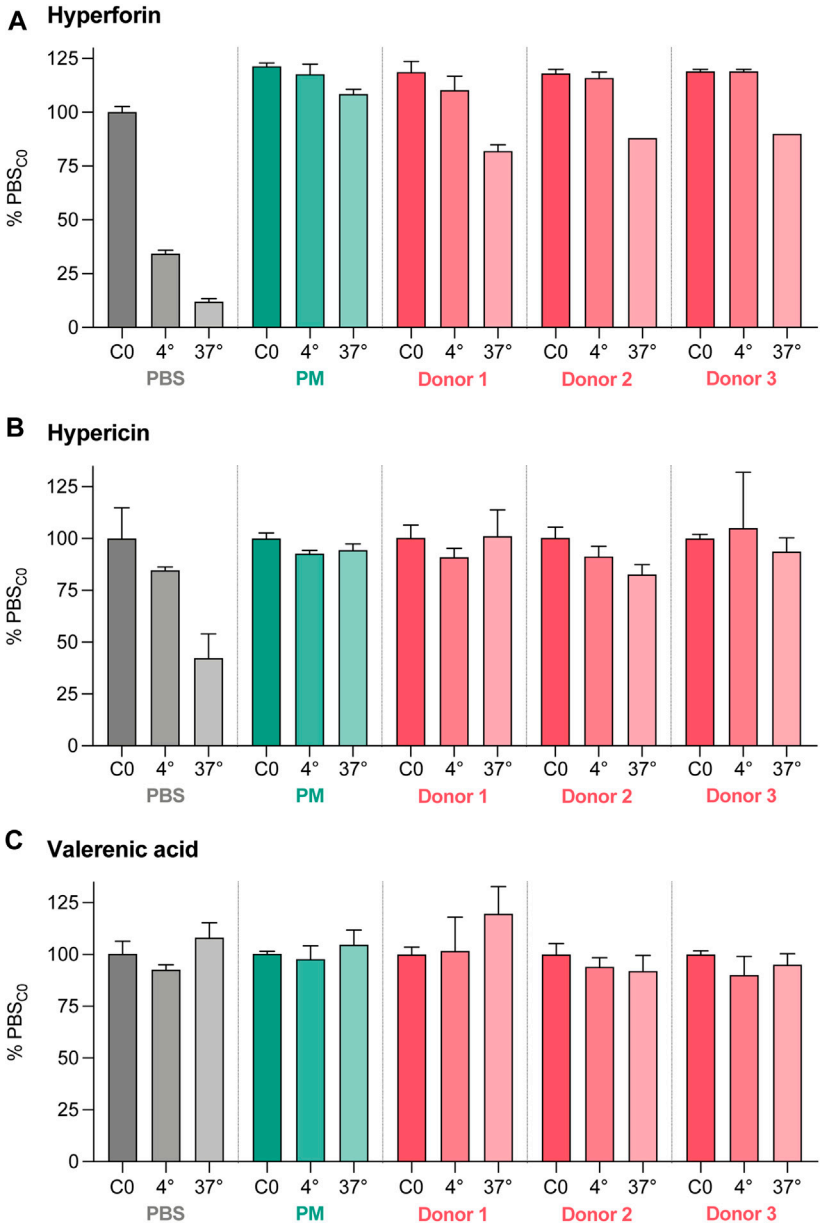

FIGURE 6
Stability data for hyperforin **(A)**, hypericin **(B)**, and valerenic acid **(C)** expressed as percentage (%) of the initial concentration (C0) in PBS. The stability test was performed for 360 min at two different temperatures (4°C and 37°C) and three different matrices (PBS), perfusion medium (PM), and placental homogenates from three different donors. Differences due to matrix effects were excluded in a separate experiment (Supplementary Figure S2). All data are represented as mean ± SD.

Figure S2). A loss of hyperforin was observed in the presence of placental homogenate, with 82.0% (Donor 1), 88.0% (Donor 2), and 90.0% (Donor 3) remaining after 360 min. Some degradation in homogenate at 37°C was also observed with hypericin (101.0%, 82.7%, and 93.7% respectively). The use of three different placental homogenates (Donors) resulted in comparable values for all test substances. Some differences attributable to matrix effects could be demonstrated for hyperforin and hypericin in separate experiments (see Supplementary Figure S2).

Effects on placental function and histology

Possible effects of the study compounds on placental metabolic activity and hormonal production were also investigated. The metabolic activity of all perfused placental tissues was similar, as neither glucose consumption nor concomitant lactate production were affected by the study compounds (Figure 7A). With antipyrine (from control perfusions), the total glucose consumption and lactate production during the perfusion were 0.39 and 0.27 µmol/g/

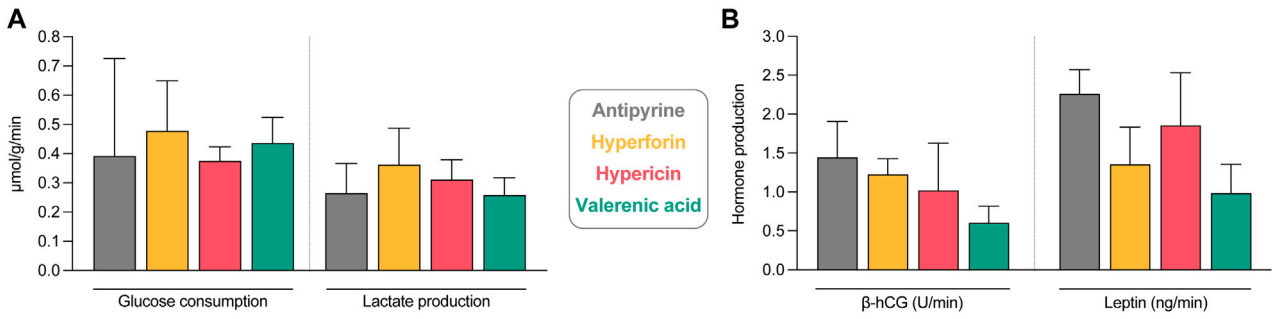

FIGURE 7
Assessment of tissue viability and functionality during the *ex vivo* human placental perfusion. **(A)** Glucose consumption and lactate production under exposure to study compounds and antipyrine (from control perfusions). Displayed are the changes between beginning and end of the perfusion in fetal and maternal circuits. Data are normalized by the total perfusion time (min) and perfused cotyledon weight (g). All data are represented as mean ± SD of three to four independent experiments. **(B)** Beta-human choriogonadotropin (*β*-hCG) and leptin tissue production of perfusions with study compounds and antipyrine (from control perfusions). Displayed is the net release rate of placental hormones during the placental perfusion, normalized by the total perfusion time (min). All data are represented as mean ± SD of three to four independent experiments (except for the leptin value of antipyrine, where only two values are included). No statistically significant differences were found between the groups ($p > 0.05$ in all cases).

min, respectively. Beta-human chorionic gonadotropin (*β*-hCG) and leptin production were determined as an additional measure for placental function and found to be somewhat lower in the presence of all compounds (Figure 7B). However, neither hyperforin, hypericin, nor valerenic acid inhibited their production in a statistically significant manner. This implied that the tissue of all placentae retained their functionality throughout the *ex vivo* perfusion period. A *β*-hCG production of 1.44 U/min and leptin production of 2.26 ng/min was observed in control perfusions with antipyrine only.

Detailed histopathological examination of the perfused tissue revealed that microscopic effects of perfusion were seen in addition to the macroscopically apparent pale tissue (Table 3): i) villous vessels of the perfused side were ≥80% empty (non-perfused area was ≤20% empty of blood), ii) intervillous space of perfused tissue was ≥70% empty of blood (vs. 30%–80% in non-perfused area) and equally or more dilated in contrast to the non-perfused side, iii) formation of hydropic villous changes was found more frequently in the perfused (5%–40%) than in the non-perfused areas (0%–5%), and iv) a clear transition between perfused and non-perfused tissue was observed in most of the cases. The endothelium in the perfused tissue was still viable after 360 min of perfusion. Other histopathological observations that argue against damage to placental tissue after perfusions with the test substances (hyperforin, hypericin, valerenic acid) were i) a low percentage of thrombi in villous vessels (up to 5%) of perfused tissue, ii) no thrombi detectable in vessels of stem villi (perfused and non-perfused), iii) trophoblast vacuolization in perfused areas occurred in a proportion of 0%–30% and was substance-independent, some but not all (two out of four) cotyledons perfused with valerenic acid showed higher proportions (80%–90%), iv) no ruptures of villous vessels, and v) no extravasations into villous stroma. No signs of inflammation were found in any of the examined perfused tissue areas, as neither bacteria nor neutrophils were present in the villous vessels and intervillous spaces. In addition, the assessment of global placental pathology was unremarkable, with no evidence of fetal/maternal vascular malperfusion, villous immaturity, chronic/acute villitis, chronic deciduitis, chorioamnionitis, and bacteria in the non-perfused areas of the placenta.

In vitro permeability assays

All results shown so far were obtained with term placentae. To better evaluate the transplacental passage of valerenic acid, the *in vitro* BeWo b30 Transwell model was used. Hyperforin and hypericin were not suited for these experiments as they did not cross the cell-free inserts to a sufficient extent (data not shown). In our hands, a dense BeWo b30 cell layer was obtained 9 days after cell seeding on semi-permeable insert membranes. This timepoint was chosen because i) translocation of NaF (a marker of paracellular passive diffusion) was minimal, with a basolateral amount of 2.8% of the initial NaF concentration (Supplementary Figure S3), ii) TER values reached a value of ≥30 Ωcm^2 on day 9 after cultivation, which was markedly higher than in previous days (Supplementary Figure S4A), and the C_{cl} was below the expected 5 µF/cm^2 (Supplementary Figure S4B) and, iii) staining of nuclei (blue) and actin (red) showed gapless growth of BeWo b30 cells on cell culture inserts (Supplementary Figure S5).

In this Transwell model, valerenic acid did not cross the placental cell layer within 60 min to reach detectable concentrations. In contrast, valerenic acid could pass the semi-permeable cell-free insert to the same extent as the positive control antipyrine (Figure 8A; 28.3 µL within 60 min). Antipyrine crossed from the apical to the basolateral compartment through the placental cell layer and the semi-permeable cell-free insert membrane at the same clearance rate (Figure 8B; 35.5 µL and 33.5 µL, respectively). The P_{app} was zero for valerenic acid, and 28.0×10^{-6} cm/s for antiyprine. The recoveries after 60 min were 73.7% ± 11.9% for valerenic acid, and 81.9% ± 14.2% for antipyrine, and included the final amounts in the apical, basolateral, and cellular compartment (Supplementary Figure S6). Mean TER and C_{cl} values were similar before and after the permeability experiment with valerenic acid and antipyrine (Supplementary Figure S7).

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TABLE 3 Detailed histopathological evaluation assessing the microscopic effects of human *ex vivo* placental perfusions with hyperforin (n = 4), hypericin (n = 3) and valerenic acid (n = 4), and the damage of placental tissue in perfused areas compared to non-perfused areas.

		Hyperforin				Hypericin			Valerenic acid			
	Experiment number	1	2	3	4	1	2	3	1	2	3	4
Microscopic effects of perfusion (in %)	Emptiness of villous vessels	95	95	90	80	90	80	85	90	95	80	80
	Emptiness of villous vessels in non-perfused	10	20	5	5	20	5	1	10	10	1	1
	Intervillous spaces without blood	70	95	99	80	90	95	95	95	95	95	95
	Intervillous spaces without blood in non-perfused	30	30	40	60	40	40	80	40	70	50	60
	Dilatated intervillous space	90	90	70	70	60	30	95	70	50	90	90
	Dilatated intervillous space in non-perfused	60	50	70	80	50	30	85	50	20	30	70
	Hydropic changes	20	5	40	10	20	10	5	0	25	40	5
	Hydropic changes in non-perfused	0	0	1	0	5	0	0	0	5	5	0
	Sharp transition perfused/non-perfused	N	Y	Y	N	Y	N	Y	N	Y	Y	Y
Damage of placental tissue (in %)	Thrombi in villous vessels	0	0	0	0	1	0	0	5	1	5	0
	if so: in non-perfused too?	—	—	—	—	ND	—	—	1	ND	1	-
	Vacuolated trophoblast in villi	10	30	10	0	20	20	5	1	90	80	5
	if so: in non-perfused too?	0	0	1	—	0	0	0	ND	0	5	0
Global placental pathology	Else	N	N	N	Intervillous thrombus <5%	CCB	N	CCB	N	Formation of artificial cavities in 10% of perfused areas	N	Singular non-occlusive thrombus in stem villus; CCB

CCB, compensatory core budding; N = no; ND = not determined; Y = yes.

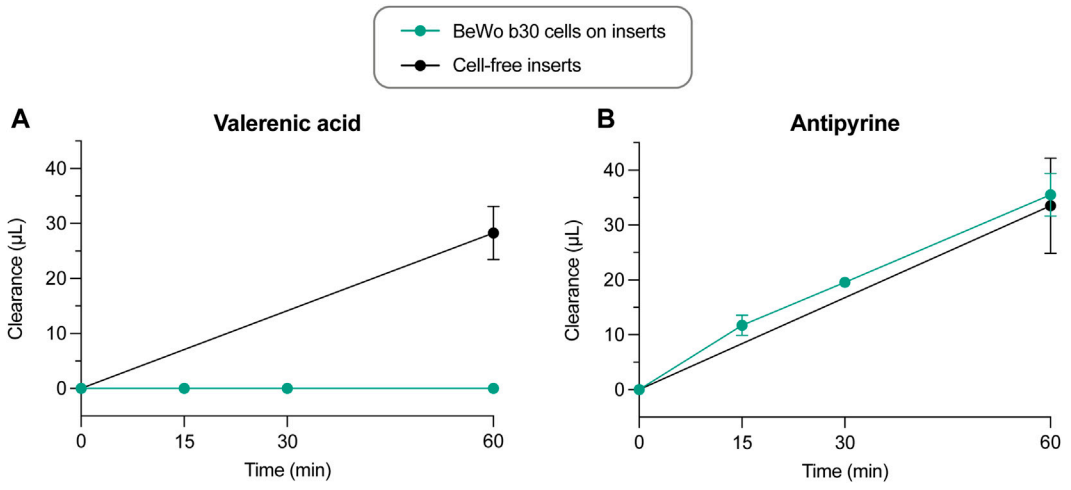

FIGURE 8
Clearance vs. time curves of control experiments across cell-free inserts (n = 4) and across BeWo b30 cell layers for valerenic acid and antipyrine (n = 3–6). Data are presented as mean ± SD.

Discussion

Main findings

Our results from the *ex vivo* human term placental perfusion model showed that only minor amounts of hyperforin were transported to the fetal circulation, resulting in a very low FM ratio. Hypericin did not cross the placental barrier, while valerenic acid equilibrated between the maternal and fetal compartments. In addition, metabolic, functional, and histopathological properties of placentae during perfusions were not significantly altered by the test substances. Observations performed with the *in vitro* Transwell model with human placental cells indicated that valerenic acid was unable to cross the cell monolayer, thereby suggesting that the compound may not cross cytotrophoblast layers.

Our observations from the *ex vivo* human term placental perfusion model fit well with the predicted physicochemical properties of the investigated molecules and an expected transport through the placenta barrier by passive diffusion. It is likely that the relatively high MW (>500) of hyperforin and hypericin, together with their ionization states at pH 7.2, hindered their transfer [compare with (Syme et al., 2004; Tetro et al., 2018)]. In contrast, the markedly smaller valerenic acid could equilibrate between maternal and fetal compartments almost as quickly as antipyrine (MWs of 234.3 and 188.2, respectively). Although our results are in line with a transfer by passive diffusion, a possible involvement of additional mechanisms in the transplacental transfer of hyperforin, hypericin and valerenic acid requires further investigation. The results of our stability experiments revealing some degradation of hyperforin, and to a smaller extent hypericin, at 37°C but not at 4°C suggest some metabolization of these compounds through placenta enzymes.

Strengths and limitations

Plant extracts consist of a variety of different compounds, some of which are present in low amounts only. Therefore, it was crucial to develop sensitive U(H)PLC-MS/MS methods capable of detecting very low concentrations of analytes that one would expect *in vivo* upon oral ingestion of phytomedicines. However, it should be noted that confident statements can only be made within the calibration range (see Materials and methods). A limitation of the study is that concentrations below the limit of quantification had to be assumed to be zero. *Ex vivo* placental perfusion is to date the only experimental model preserving the structural integrity and cell-cell organisation of the organ. It most closely mimics the *in vivo* situation and, therefore, provides good predictions for placental transfer *in vivo*. A disadvantage of the model is that it represents the situation at term, when transplacental transfer is known to be maximal. For compounds that were not transferred in this model (hyperforin and hypericin) one can reasonably assume that they are also not transferred at earlier stages of pregnancy. The *in vitro* Transwell model that mimics the cytotrophoblast monolayer (Bode et al., 2006; Vähäkangas and Myllynen, 2006), provided valuable information for valerenic acid. We opted for not testing hyperforin and hypericin in the Transwell model, since under our experimental conditions these molecules did not cross the membranes of the inserts (in the absence of a cell layer) in a measurable way. The transfer across the membranes was not significantly increased when using high protein concentration in the medium (up to 4% BSA; data not shown). High protein concentrations have been used to improve solubility and, hence, transfer of poorly soluble compounds (Füller et al., 2018). Similar limitations of such permeability experiments with hypericin have been previously described in the Caco cell model (Verjee et al., 2015). A limitation common to both models is that they cannot fully represent the *in vivo* situation, as they do not take into account aspects such as dissolution, absorption, distribution, metabolism, and excretion of the compounds (Poulsen et al., 2009; Hutson et al., 2011; Myllynen and Vahakangas, 2013), which strongly influence transplacental transfer. Finally, the two methods only allow a study of short-term toxic effects on placental tissue and cells, while in pregnancy the placentae are exposed for extended periods to the substances. Especially with compounds that accumulate in cell membranes, such as hypericin [(Verjee et al., 2015); own unpublished observations], this might lead to an underestimation of possible undesired effects.

Recovery of hyperforin, hypericin and valerenic acid

Experiments with hyperforin and hypericin required special attention, due to their high lipophilicity and poor solubility. In the absence of biological material, hyperforin showed a significant loss in the empty perfusions (65.4% over a 4-h period) which could be due to adsorption to tubing/equipment or precipitation. The higher recovery in the presence of placental tissue could be explained by the higher protein content in the system. The presence of protein is needed to stabilize and solubilize this compound, as described in literature (Füller et al., 2018), and this was reflected by the high stability in the PM but not in presence of PBS (Figure 6). The amounts in the fetal and maternal compartments, and the amounts removed by sampling were comparable for hyperforin (78.4%) and for antipyrine (79.2%). In addition, loss of hyperforin in the incubations with placental homogenates may indicate a possible metabolisation. The lowest final recovery was found for hypericin (54.0%), although there was only a small loss due to system adsorption (3.8% over a 4-h period). Again, stability was good in PM but not in PBS (Figure 6). However, the recovery data did not take into account the percentage in placental tissue. Interestingly, fluorescence microscopic images showed a considerable accumulation of hypericin in placental cells (data not shown), which is similar to previous observations with Caco-2 cells (Verjee et al., 2015). In the placental perfusion model valerenic acid showed good stability and high recovery, thereby facilitating data interpretation.

St. John's wort: Comparison with previous *in vitro* studies

Previous *in vitro* studies showed that extracts from St. John's wort had no negative impact on placental cells at concentrations up to 30 μg/mL (cytotoxicity, apoptosis) or 100 μg/mL (genotoxicity, metabolic activity, and influence on placental cell differentiation) (Spiess et al., 2021). Our present data with term placentae suggest

that a possible fetal exposure to hypericin and hyperforin is likely minimal. This is particularly important, as transplacental transport is maximal at term due to a decrease of cell layer thickness and number of cell layers towards the end of pregnancy (van der Aa et al., 1998; Vähäkangas and Myllynen, 2006). *In vitro*, hyperforin showed no effects on viability, metabolic activity, and on induction of placental cell differentiation at concentrations up to 30 μM. However, hyperforin led to increased apoptosis and genotoxic effects starting at concentrations of 3 and 10 μM, respectively, and inhibited FSK-induced placental cell differentiation at concentrations of ≥1 μM (Spiess et al., 2022b). It should be noted that these test concentrations were significantly higher than reported plasma concentrations in humans. Upon oral administration of a single dose of 300 mg St. John's wort extract containing 14.8 mg hyperforin a maximum plasma concentration of 150 ng/mL (approx. 0.28 μM) was reached (Biber et al., 1998). C_{max} values of 83.5 ng/mL (≙ 0.16 μM) and 122 ng/mL (≙ 0.23 μM) hyperforin were determined after single dose administration of 612 mg and 900 mg dry extract, respectively (Schulz et al., 2005b; a). However, the hyperforin content in commercially available products can vary considerably (Schäfer et al., 2019). Given that low amounts of hyperforin can possibly cross the transplacental barrier, and our recent data on inhibition of cell differentiation at ≥ 1 μM concentrations in BeWo cells (Spiess et al., 2022b), it may be prudent to resort in pregnancy to products with a low hyperforin content. Hypericin lowered the viability of placental cells already at 1 μM concentrations, and apoptotic and genotoxic effects were seen at concentrations of 1 and 10 μM, respectively (Spiess et al., 2022b). The amount of hypericin in commercially available drugs varies. For products marketed in Switzerland and Germany, amounts ranging from 0.08 mg to 0.21 mg per 100 mg of tablet have been reported (Schäfer et al., 2019). In human volunteers, plasma concentrations of 2.2 ng/mL hypericin (≙ 4.36 nM) have been found (Jackson et al., 2014). The plasma levels of hypericin thus are significantly lower than the concentrations found toxic in BeWo cells (Spiess et al., 2022b). However, plasma levels of hypericin may increase upon co-administration of certain other drugs (Jackson et al., 2014).

Valerian: Comparison with previous *in vitro* studies

At concentrations up to 30 μg/mL, no signs of cytotoxicity or apoptosis, and at concentrations up to 100 μg/mL, no signs of genotoxicity, alteration of metabolic activity and placental cell differentiation were observed in BeWo cells for the valerian extract (Spiess et al., 2021). Valerenic acid was not permeable in the *in vitro* Transwell model mimicking continuous cytotrophoblast layers that are likely to play a role in the placenta barrier at early stages of pregnancy. However, it reached an equilibrium between the fetal and the maternal circulation in the *ex vivo* placental perfusion model representative for late stages of pregnancy. With respect to valerenic acid, concentrations up to 30 μM did not lower viability of BeWo b30 cells, and no increase in apoptosis or genotoxicity, and no negative effect on the metabolic activity and cell differentiation were observed (Spiess et al., 2022b). Valerenic acid contents ranging from 1.21 mg/g to 2.46 mg/g product have been reported in products marketed in Australia (Shohet et al., 2001), while 0.57 mg–2.20 mg valerenic acid per tablet/capsule have been found in products marketed in Switzerland (Winker et al., manuscript under review). For valerenic acid, maximal plasma concentrations of 2.3 ng/mL (≙ 9.82 nM) to 3.3 ng/mL (≙ 14.08 nM) have been reported (Anderson et al., 2005; Anderson et al., 2010). Considering that valerenic acid did not affect cell viability in BeWo b30 cells at concentrations up to 30 μM (Spiess et al., 2022b), the valerenic acid content in products, and reported plasma concentrations, there appears to be a large safety margin.

Final statement

Hyperforin could only cross the complex placental barrier to a very small extent, while hypericin appeared to be non-permeable. Valerenic acid crossed the placental barrier at term when permeability is higher, but not in the *in vitro* BeWo transfer model representative of cytotrophoblast monolayer. Taken together, our data suggest that when treating mild mental disorders with St. John's wort and valerian extracts, fetal exposure to hypericin and hyperforin and, at early stages of pregnancy to valerenic acid, is likely to be low. Our study included so far only single compounds that are considered as relevant for the pharmacological properties of St. John's wort and valerian. Given that the entire extracts, and not just single compounds, are considered as the active ingredient of phytomedicines, the possible influence of the extract matrix on placental permeability of these compounds should be evaluated. Recent *in vitro* data in BeWo cells with St. John's wort and valerian extracts, and with hyperforin, hypericin and valerenic acid (Spiess et al., 2021; Spiess et al., 2022b) suggest moreover no toxicity at concentrations to be expected in humans at the recommended extract doses, but caution against using products containing high amounts of hyperforin.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Canton of Zurich (KEK-StV73 Nr. 07/07; 21 March 2007). The patients/participants provided their written informed consent to participate in this study.

Author contributions

DS performed the *ex vivo* placental perfusion and *in vitro* Transwell experiments, performed data analysis, interpretation, and visualisation, and wrote parts of the draft manuscript. VA developed bioanalytical methods, performed all bioanalyses, and wrote parts of the manuscript. AC developed bioanalytical methods.

JR was involved in the establishment of the *in vitro* model and helped with all permeability experiments. AT conducted stability testing, performed data analysis, and supervised the bioanalytical method development, together with MO and SK helped DS with data analysis and paper editing. MR was responsible for the histopathological examinations. APS-W, MH, and OP designed the study and supervised DS, VFA and AC, respectively. All authors were involved in data interpretation and reviewing of the manuscript and agreed with the final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1123194/full#supplementary-material

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Chapter 5 – Supporting Information

Placental passage of hyperforin, hypericin, and valerenic acid

Deborah Spiess*, Vanessa Fabienne Abegg*, Antoine Chauveau, Joshua Rath, Andrea Treyer, Michael Reinehr, Sabrina Kuoni, Mouhssin Oufir, Olivier Potterat, Matthias Hamburger, Ana Paula Simões-Wüst

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* *These authors contributed equally to the work and should be considered as joint first authors*

Supplementary Figures

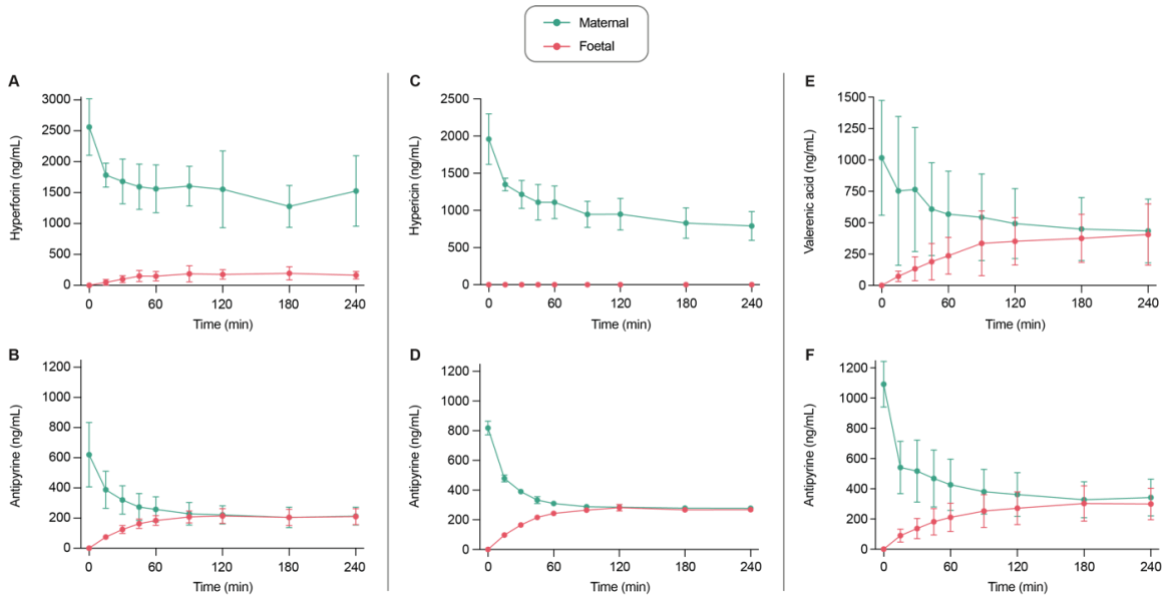

Figure 1S. *Ex vivo* human placental perfusion profiles of hyperforin (**A**), hypericin (**C**) and valerenic acid (**E**) with corresponding connectivity control (antipyrine) transfers (**B**, **D** and **F**, respectively). Concentrations are expressed as absolute concentrations in ng/mL (● maternal and ● foetal). All values are expressed as mean ± SD of three to four independent experiments.

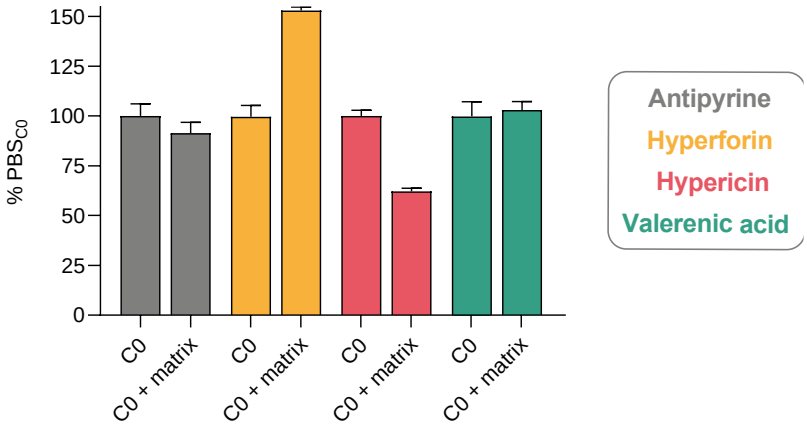

Figure 2S. Matrix effects by the homogenate on the substances antipyrine, hyperforin, hypericin, and valerenic acid. The compounds were spiked into PBS and then diluted with either an equal volume of PBS (C0) or an equal volume of tissue homogenate (C0 + matrix). The samples were processed for LC-MS analysis within 1-3 min after the dilution to avoid interference of any stability issues. All values are expressed as mean ± SD of three to four independent experiments.

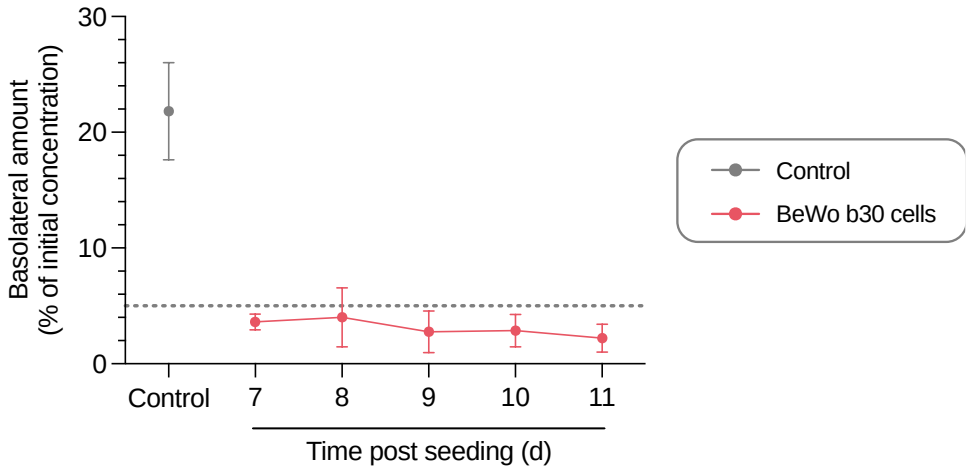

Figure 3S. Evaluation of monolayer formation using a sodium fluorescein (NaF) exclusion assay. The y-axis displays the amount of NaF in the basolateral compartment as percent (%) of initial concentration (5 µM) after 60 min. Control consists of a cell-free insert only (n=3). Data with cells represents the mean ± standard deviation of 3 biologically independent experiment with three to four technical replicates.

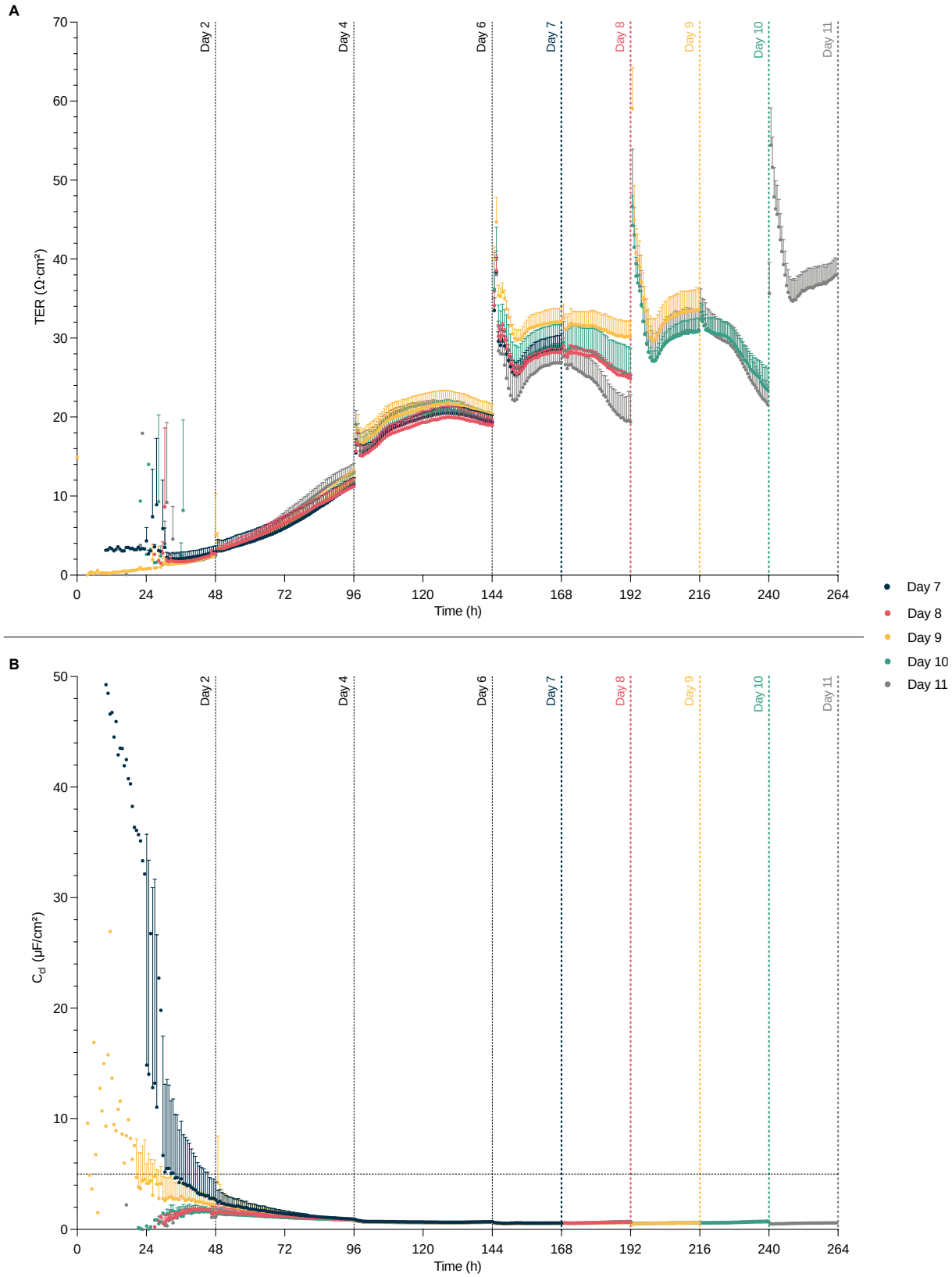

Figure 4S. Transepithelial electrical resistance (TER, **A**) and electrical capacitance C_{cl} (**B**) values evaluating the monolayer formation of BeWo b30 cells grown on Transwell® polycarbonate membrane insets. Cell culture medium was changed every other day (days 2, 4, 6, 8, and 10, respectively). Each day includes data (mean ± SD) from a biologically independent experiment with four technical replicates, with four inserts removed daily (starting from day 7) to perform a sodium fluorescein (NaF) exclusion assay.

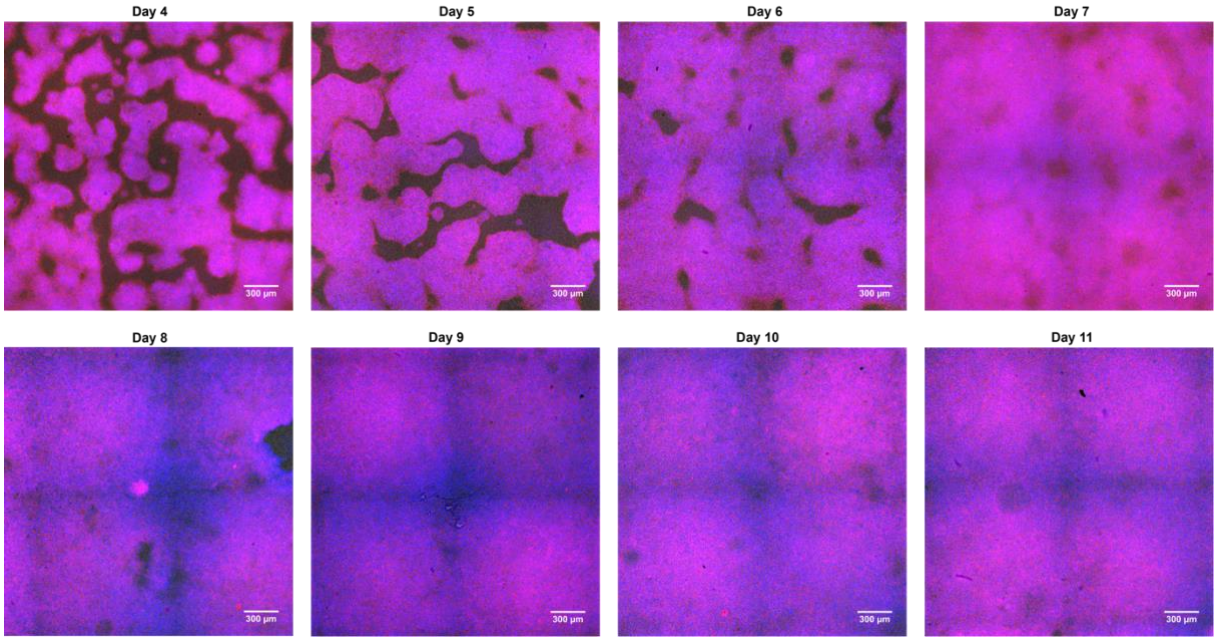

Figure 5S. Staining of nuclei (blue) and actin (red) of BeWo b30 cells growing on cell culture inserts after 4-11 days of cultivation. The images are representative of two independent cultures. A total of 49 individual tiles were acquired and automatically stitched to an overall image of the membrane, with only a selection of four tiles of the center shown here.

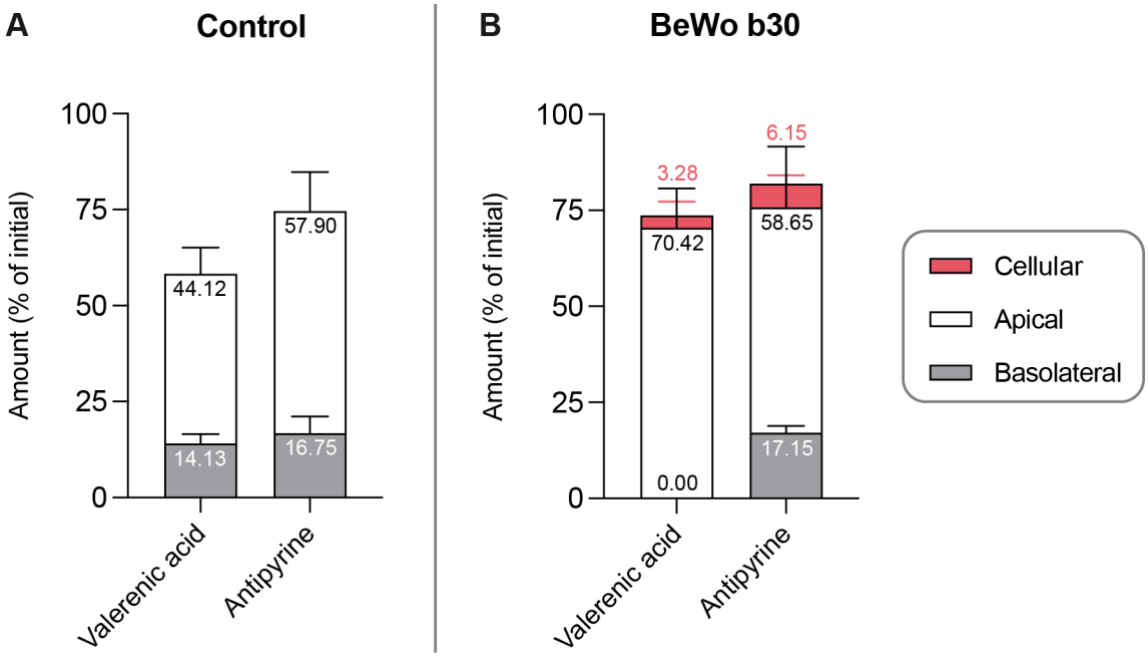

Figure 6S. Recoveries of valerenic acid and antipyrine using cell-free inserts (control, **A**) and BeWo b30 cells on inserts (**B**). The y-axis displays the amount of valerenic acid or antipyrine in the apical, basolateral, or cellular compartment as percent (%) of initial concentration (5 µM) after 60 min. Control consists of a cell-free insert only (n=4), which was pre-incubated with cell culture medium for 9 days to match the experimental conditions of the Transwell experiments with BeWo b30 cells (n=3-6). Data with cells represents the mean ± standard deviation of three biologically independent experiment with three technical replicates.

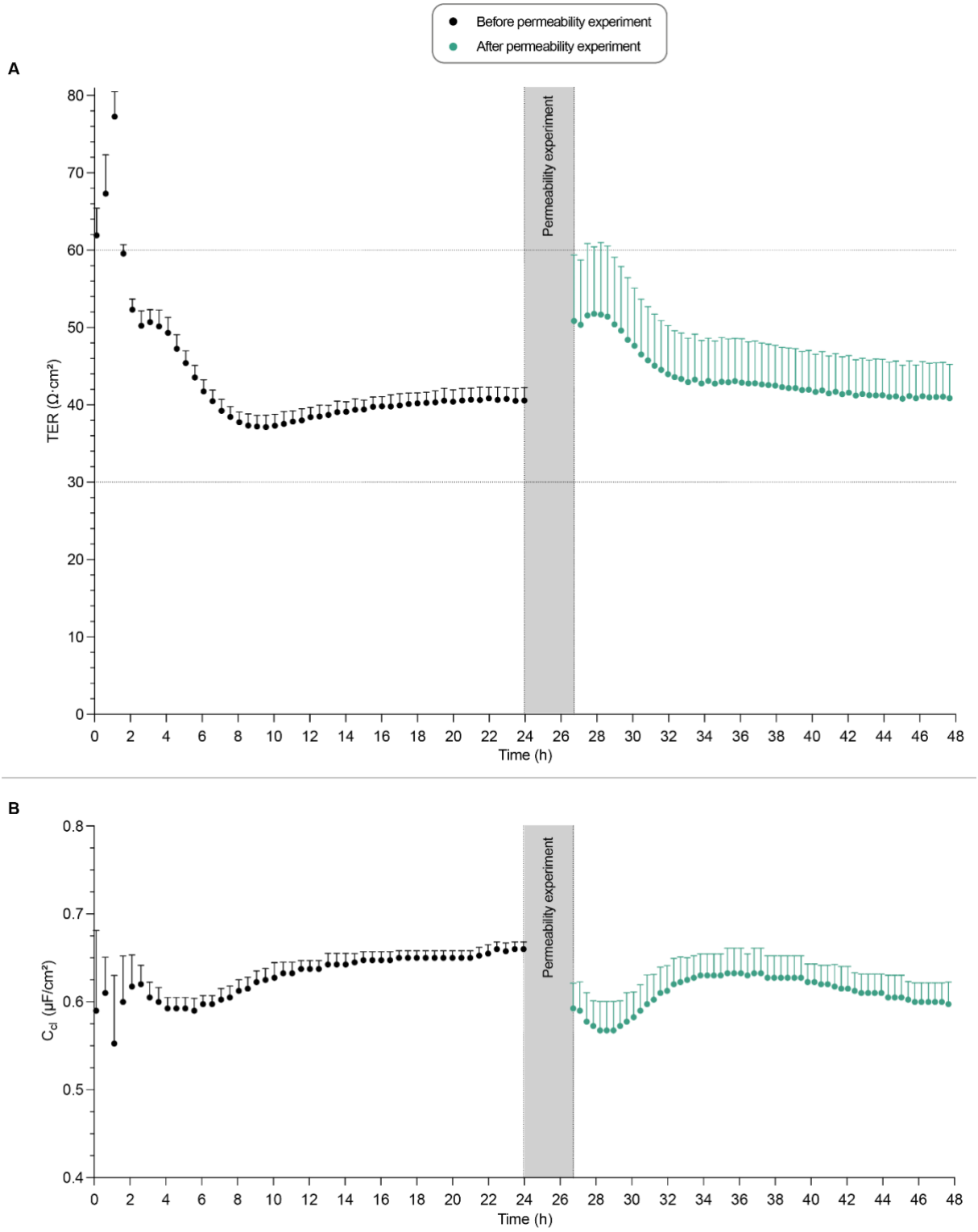

Figure 7S. Development of transepithelial electrical resistance (TER, **A**) and electrical capacitance C_{cl} (**B**) values. Measurements started on day 8 of cultivation and simultaneously 24 h prior to the BeWo b30 exposure to valerenic acid and antipyrine. The measurement was paused during the permeability experiment (day 9 of cultivation) and recorded for another 24 h after exposure. Each data set is representative of a biologically independent experiment with four technical replicates.

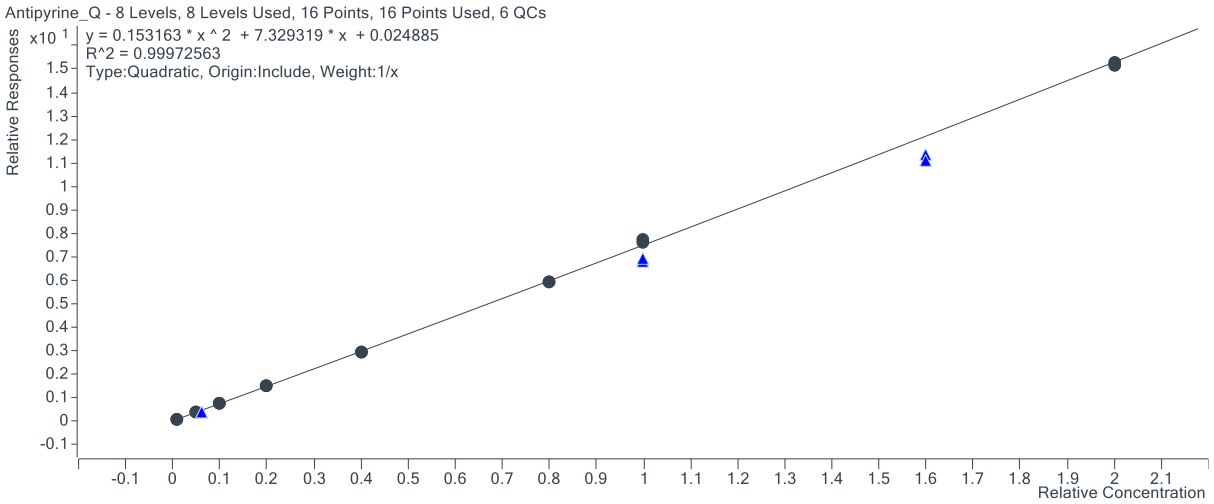

Figure 8S. Calibration curve of antipyrine (calibrators are shown as circles, quality controls [QCs] are shown as triangles).

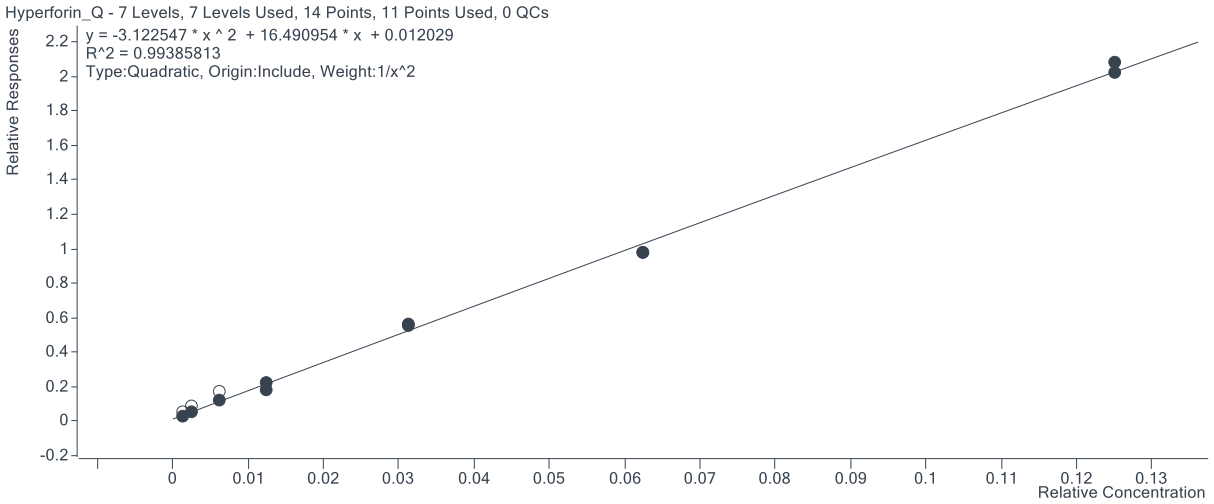

Figure 9S. Calibration curve of hyperforin (calibrators are shown as circles where open circles are excluded values and closed circles are included values).

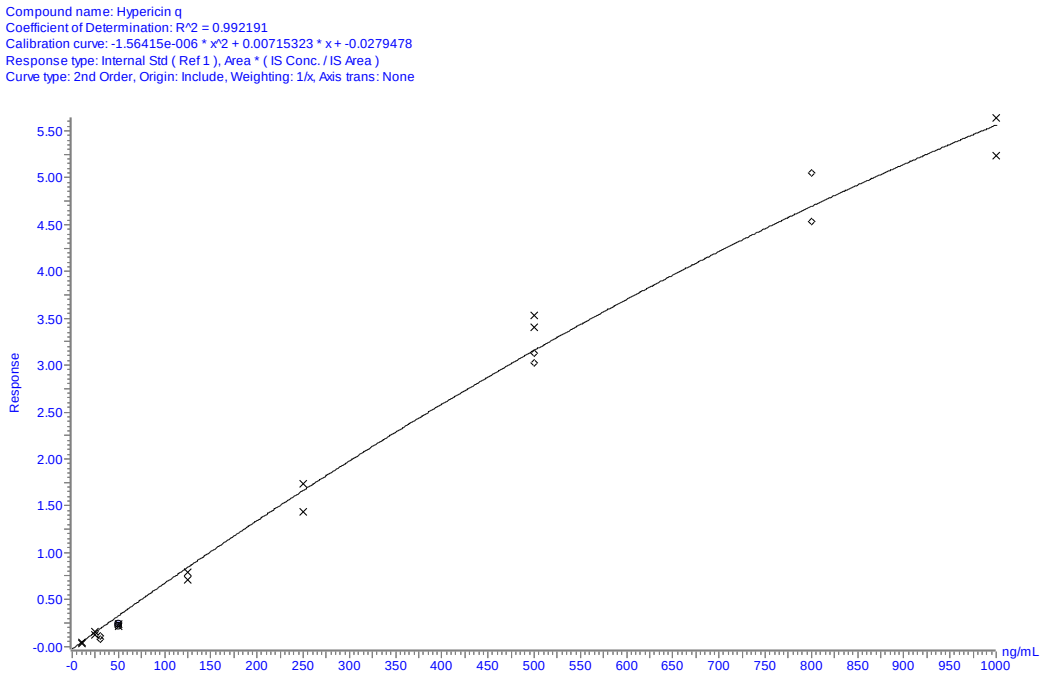

Figure 10S. Calibration curve of hypericin (calibrators are shown as x and quality controls [QCs] are shown as diamonds).

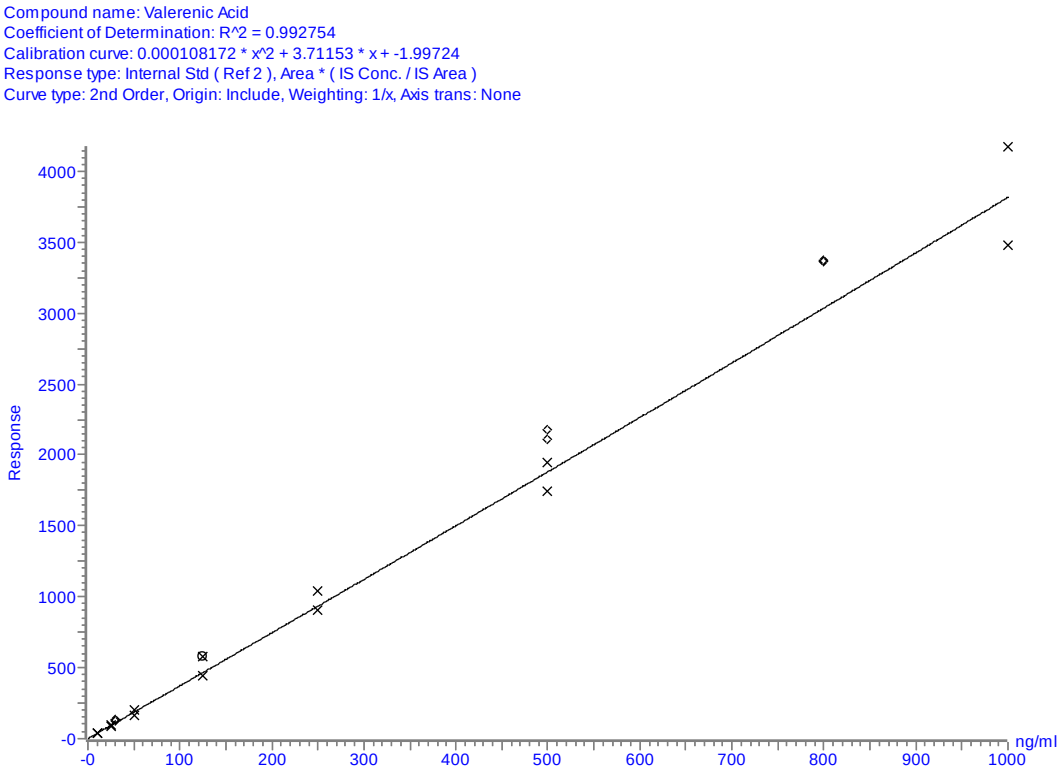

Figure 11S. Calibration curve of valerenic acid (calibrators are shown as x and quality controls [QCs] are shown as diamonds).

Supplementary Tables

Table 1S. Recovery of study compounds after 240 min of *ex vivo* human placental perfusion in relative amounts (% found) ± SD in different compartments.

	M perfusate (% found)	F perfusate (% found)	Sampling (% found)	Final recovery* (% found)
Antipyrine	29.5 ± 2.4	27.8 ± 0.8	21.8 ± 1.3	79.2 ± 2.6
Hyperforin	52.2 ± 21.5	5.3 ± 1.8	21.0 ± 4.5	78.4 ± 23.0
Hypericin	36.7 ± 11.0	0.1 ± 0.1	17.2 ± 2.7	54.0 ± 13.7
Valerenic acid	34.4 ± 9.8	33.1 ± 10.7	24.1 ± 4.8	91.5 ± 19.1

M = maternal; F = foetal; * sum of compound present in foetal and maternal perfusates at the end of a perfusion and the amount sampled during the perfusion (sampling from foetal and maternal perfusates).

Table 2S. Characteristics of placentae used, and data from individual perfusions with all study compounds (hyperforin, n=4; hypericin, n=3; and valerenic acid, n=4).

	Hyperforin				Hypericin			Valerenic acid			
Experiment Nr	1	2	3	4	1	2	3	1	2	3	4
Placenta weight (g)	570	470	730	520	515	630	490	460	570	460	550
Cotyledon weight (g)	17.21	7.93	6.68	17.38	14.48	14.97	21.26	10.87	6.69	27.08	13.26
Volume loss* (M, mL)	2	6	-2	2	0	-4	-3	3	9	-3	0
Volume loss* (F, mL)	6	16	8	1	15	13	8	0	9	16	5
Preparation time° (min)	30	19	15	14	18	16	20	14	16	17	14
Cannulation time§ (min)	30	14	24	25	13	15	18	36	ND	18	23
Open pre-phase (min)	20	20	20	20	20	20	20	20	20	20	20
Close pre-phase (min)	20	20	20	20	20	25	20	20	20	20	21
Perfusion time (min)	240	240	240	240	240	240	240	240	240	240	240

* Total volume loss at the end of the perfusion time; M = maternal; F = foetal; ° time from birth to begin of cannulation; § time from cannulation to begin of open pre-phase; ND = not determined.

Table 3S. U(H)PLC gradients and flow rate for antipyrine, hyperforin, hypericin, and valerenic acid. For antipyrine and hyperforin, the mobile phase consisted of A1 (purified water with 0.1% formic acid) and B1 (MeCN with 0.1% formic acid). For hypericin and valerenic acid, the mobile phase consisted of A2 (purified water with 0.1% NH_4OH at pH 10.7) and B2 (MeCN/purified water with 0.1% NH_4OH, ratio 9:1)

Antipyrine			
Time (min)	**A1 (%)**	**B1 (%)**	**Flow rate (mL/min)**
0.00	100.0	0.0	
0.50	100.0	0.0	
3.00	37.89	62.11	
3.01	0.0	100.0	0.4
4.00	0.0	100.0	
4.01	100.0	0.0	
5.00	100.0	0.0	
Hyperforin			
Time (min)	**A1 (%)**	**B1 (%)**	**Flow rate (mL/min)**
0.00	100.0	0.0	
1.00	100.0	0.0	
2.00	0.0	100.0	0.4
3.80	0.0	100.0	
3.81	100.0	0.0	
5.00	100.0	0.0	
Hypericin			
Time (min)	**A2 (%)**	**B2 (%)**	**Flow rate (mL/min)**
0.00	95.0	5.0	
1.00	95.0	5.0	
6.00	30.0	70.0	
6.10	0.0	100.0	0.4
7.00	0.0	100.0	
7.10	95.0	5.0	
8.00	95.0	5.0	
Valerenic acid			
Time (min)	**A2 (%)**	**B2 (%)**	**Flow rate (mL/min)**
0.00	95.0	5.0	
1.00	95.0	5.0	
6.00	30.0	70.0	0.4
6.01	0.0	100.0	
7.00	0.0	100.0	
8.00	0.0	100.0	

Table 4S. Calibrators and calibration curve parameters for the determination of antipyrine. Response: $A \times Conc^2 + B \times Conc + C$, 1/X weighting, Quadratic regression, included origin (n=12).

	Concentration (ng/mL)								Regression parameters			
	5	**25**	**50**	**100**	**200**	**400**	**500**	**1000**	**A**	**B**	**C**	**R^2**
Mean	5.236	24.358	50.440	101.986	199.923	393.158	508.523	979.559	6.98E-01	8.090	0.054	0.999
SD	0.446	0.584	2.912	5.947	8.119	8.690	20.043	63.632	1.71E+00			
CV%	8.521	2.396	5.772	5.831	4.061	2.210	3.941	6.496				
RE%	4.730	-2.568	0.879	1.986	-0.039	-1.711	1.705	-2.044				

Table 5S. Calibrators and calibration curve parameters for the determination of hyperforin. Response: $A \times Conc^2 + B \times Conc + C$, $1/X^2$ weighting, Quadratic regression, included origin (n=6).

	Concentration (ng/mL)							Regression parameters			
	2.5	**5**	**12.5**	**25**	**62.5**	**125**	**250**	**A**	**B**	**C**	**R2**
Mean	2.424	5.283	12.631	25.229	62.450	117.365	256.008	1.18E+01	11.013	0.006	0.989
SD	0.094	0.151	1.131	2.692	4.936	2.031	17.714	1.34E+01			
CV%	3.861	2.862	8.958	10.671	7.904	1.730	6.919				
RE%	-3.033	5.657	1.045	0.917	-0.081	-6.108	2.403				

Table 6S. Calibrators and calibration curve parameters for the determination of hypericin. Response: $A \times Conc^2 + B \times Conc + C$, 1/X weighting, Quadratic regression, included origin (n=6).

	Concentration (ng/mL)							Regression parameters			
	10	**25**	**50**	**100**	**250**	**500**	**1000**	**A**	**B**	**C**	**R^2**
Mean	10.217	24.550	47.825	121.633	249.400	513.250	992.567	-7.49E-06	0.744	0.874	0.994
SD	1.105	2.270	3.530	9.308	18.055	48.067	71.287	1.15E-05			
CV%	10.819	9.248	7.382	7.652	7.239	9.365	7.182				
RE%	2.167	22.750	-4.350	21.633	-0.240	2.650	-0.743				

Table 7S. Calibrators and calibration curve parameters for the determination of valerenic acid. Response: $A \times Conc^2 + B \times Conc + C$, 1/X weighting, Quadratic regression, included origin (n=8).

	Concentration (ng/mL)							Regression parameters			
	10	**25**	**50**	**100**	**250**	**500**	**1000**	**A**	**B**	**C**	**R^2**
Mean	10.150	23.967	50.129	122.657	255.588	496.313	1000.343	8.02E-05	2.331	-0.881	0.993
SD	0.695	1.433	4.555	11.075	27.297	43.344	58.347	1.81E-04			
CV%	6.847	5.981	9.087	9.029	10.680	8.733	5.833				
RE%	1.500	19.833	0.257	22.657	2.235	-0.738	0.034				

Table 8S. Quality control samples of antipyrine at low (QCL), medium (QCM), and high (QCH) levels (n=12).

	QCL 30	QCM 500	QCH 800
Mean	27.592	497.772	827.402
SD	0.735	28.970	59.383
CV%	2.663	5.820	7.177
RE%	-8.027	-0.446	3.425

Table 9S. Quality control samples of hyperforin (n=2).

	QCL 7.5	QCM 125	QCH 200
Mean	7.092	139.810	210.736
SD	-	4.071	12.660
CV%	-	2.912	6.007
RE%	-5.444	11.848	5.368

Table 10S. Quality control samples of hypericin (n=6).

	QCL 30	QCM 500	QCH 800
Mean	30.967	487.783	818.800
SD	3.493	15.674	55.443
CV%	11.281	3.213	6.771
RE%	3.222	-2.443	2.350

Table 11S. Quality control samples of valerenic acid (n=8).

	QCL 30	QCM 500	QCH 800
Mean	30.100	495.171	803.738
SD	2.435	34.844	62.814
CV%	8.089	7.037	7.815
RE%	0.333	-0.966	0.467

Table 12S. Carry-over assessment for antipyrine (see Materials and Methods, LC-MS/MS analysis [1]; LLOQ = lower limit of quantification; IS = internal standard).

Run	Replicate	Peak area (cts)				Individual carry-over (%)		Mean carry-over (%)	
		Blank sample		LLOQ					
		Analyte	IS	Analyte	IS	Analyte	IS	Analyte	IS
1	1	178.51	393.50	8873.33	102852.16	2.01	0.38	1.20	0.39
	2	28.99	345.72	7304.05	85516.75	0.40	0.40		
2	1	698.64	52.30	9365.15	85714.84	7.46	0.06	6.94	0.24
	2	636.10	390.00	9908.07	91407.39	6.42	0.43		
3	1	3515.76	301.60	10991.17	110887.04	31.99	0.27	19.79	0.18
	2	729.93	92.20	9610.16	109394.35	7.60	0.08		
4	1	755.22	87.74	10547.34	94071.40	7.16	0.09	11.60	0.33
	2	7840.21	729.76	48904.81	130372.79	16.03	0.56		
5	1	1617.47	93.09	12237.63	117057.68	13.22	0.08	17.78	0.13
	2	2851.55	214.78	12761.11	117699.58	22.35	0.18		
6	1	8209.80	174.88	29676.75	102812.32	27.66	0.17	-	0.22
	2	5990.02	172.57	14188.00 *	63909.47	-	0.27		
*Analyte outside acceptance criteria, carry over not calculated.						**Mean carry over**		8.19	0.16

Table 13S. Carry-over assessment for hyperforin.

Run	Replicate	Peak area (cts)				Individual carry-over (%)		Mean carry-over (%)	
		Blank sample		LLOQ					
		Analyte	IS	Analyte	IS	Analyte	IS	Analyte	IS
1	1	200.80	38.89	2999.13	93244.19	6.70	0.04	-	-
	2	382.24	34.22	8107.83*	154775.14*	-	-		
2	1	243.15	117.53	1226.37	86029.54	19.83	0.14	22.05	0.13
	2	515.77	204.69	2125.54	157231.96	24.27	0.13		
3	1	118.44	242.55	1226.37	86029.54	9.66	0.28	7.08	0.16
	2	109.48	77.16	2437.34	188650.41	4.50	0.04		
*Analyte outside acceptance criteria, carry over not calculated.						**Mean carry over**		14.56	0.15

Table 14S. Carry-over assessment for hypericin.

Run	Replicate	Peak area (cts)				Individual carry-over (%)		Mean carry-over (%)	
		Blank sample		LLOQ					
		Analyte	IS	Analyte	IS	Analyte	IS	Analyte	IS
1	1	1.07	0.00	29.10	649.46	3.68	0.00	2.82	0.00
	2	0.45	0.00	22.99	666.85	1.95	0.00		
2	1	0.00	0.00	28.57	-	0.00	-	5.07	#DIV/0!
	2	5.51	0.00	54.37	-	10.13	-		
3	1	21.01	0.22	91.60	2066.26	22.94	0.01	23.04	0.01
	2	20.22	0.00	87.37	2286.41	23.14	0.00		
						Mean carry over		14.05	0.00

Table 15S. Carry-over assessment for valerenic acid.

Run	Replicate	Peak area (cts)				Individual carry-over (%)		Mean carry-over (%)	
		Blank sample		LLOQ					
		Analyte	IS	Analyte	IS	Analyte	IS	Analyte	IS
1	1	0.00	0.00	13.02*	1481.91*	-	-	-	-
	2	0.00	0.00	34.05	1888.26	0.00	0.00		
2	1	0.00	0.00	33.51	1415.78	0.00	0.00	0.00	0.00
	2	0.00	0.00	35.89	1565.70	0.00	0.00		
3	1	0.00	1.48	37.76	1102.88	0.00	0.13	0.00	0.07
	2	0.00	0.00	32.23	1382.67	0.00	0.00		
4	1	0.15	0.00	29.41	751.35	0.50	0.00	-	-
	2	0.00	0.00	15.34*	631.10*	-	-		
*Analyte outside acceptance criteria, carry over not calculated.						**Mean carry over**		0.00	0.03

Reference

1. Spiess D, Abegg VF, Chaveau A, Treyer A, Oufir M, Reinehr M, Duong E, Potterat O, Hamburger M, and Simoes-WuestAP (2023). Placental passage of protopine in an *ex vivo* human perfusion system. *Planta-Med.* doi: 10.1055/a-1829-9546

5 FINAL REMARKS AND PERSPECTIVES

There are several reasons for the use of phytomedicines in pregnancy: on the one hand, many women are affected by NMDs [1], whereby conventional synthetic drugs do not have an optimal side effect profile, neither for the unborn child nor for the expectant mother [2-4]. On the other hand, many women prefer to use herbal alternatives [5], which are also preferentially prescribed by health care professionals [6]. Therefore, it was of great importance to investigate the safety of possible herbal alternatives in pregnancy. We have focused on St. John's wort, California poppy, valerian, lavender, and hops, which are all well-regarded phytomedicines with potential to treat NMDs. In this section, the clinical relevance of our findings for the assessment of their safety are discussed in light of physiologically attainable plasma concentrations. Having worked extensively on the safety of diverse herbal medicines, several new perspectives have emerged, which are presented thereafter.

St. John's wort (*Hypericum perforatum*)

St. John's wort has a long history as an antidepressant. For ailing pregnant mothers, it is one of the most commonly used herbal medicine, although institutions disagree on its use in pregnancy: while the EMA does not recommend its use due to lack of sufficient data [7], Embryotox considers its use acceptable, taking into account the potential for interaction, and synthetic antidepressants (e.g., sertraline, citalopram) are mentioned as more suitable alternatives [8].

According to our survey, a large majority of women use herbal pharmaceutical medicines and teas during pregnancy. Of these, only 3.5% (12/341) of pregnant women took St. John's wort. However, the use of antidepressants decreased compared with the pre-pregnancy period, and only a few women reported the use of synthetic antidepressants. Overall, a substantial number of women suffered from depressed mood, leading us to the following hypothesis: women are more likely to resort to herbal alternatives, leave symptoms of depression untreated, or conceal the use of antidepressants. The latter may be due to the stigma and negative attitudes of society towards antenatal depression and antidepressant use during pregnancy [9]. In our survey, the effectiveness of St. John's wort was only perceived moderate to good, which is surprising given that the antidepressant effect of St. John's wort has been demonstrated in several clinical trials [10].

In BeWo b30 cells, St. John's wort extract showed neither cytotoxic nor genotoxic effects on placental cells ($\leq$ 30 µg/mL) and was not able to alter their metabolic function nor affect differentiation and protein expression. The absence of toxic effects of St. John's wort extract in this concentration range was confirmed in another immortalized human placental cell line (JEG-3), as no effects on cell viability were observed at concentrations up to 100 µg/mL [11]. We suspected that a few characteristic compounds in St. John's wort *per se* might cause safety issues at

high concentrations. We focused on hyperforin and hypericin, the best-studied constituents of St. John's wort that also play a major role in pharmacological effects as two examples for closer examination [12].

Looking at the *in vitro* effects of hyperforin, the inhibition of placental cell differentiation from as low as 1 μM is particularly striking. Such inhibition would prevent the successful formation and expansion of the syncytiotrophoblast and thus significantly impair the function of the human placenta and preservation of a healthy pregnancy [13]. Under the influence of 0.3 μM hyperforin, we did not observe any negative effects on the placental cell line. The concentration of 1 μM appears relatively unattainable compared to the maximum achievable hyperforin plasma concentrations of 0.3 μM ever reported in literature (≙ 168.35 ng/mL hyperforin after administration of a soft gelatin capsule containing 300 mg St. John's wort dry extract with 5% hyperforin and 0.3% hypericin, Hammer Pharma SpA [14]). Nevertheless, the observed effects on cell differentiation conclusively show that no attempts should be made to treat pregnant women with hyperforin-enriched preparations. In fact, low hyperforin-preparations should be preferred. A variety of pharmaceutical products containing St. John's wort extract are marketed in Switzerland, with different hyperforin (and hypericin) contents, of which products (HyperiMed, Hyperiforce; A.Vogel) do not contain any hyperforin [15]. Regarding hypericin, the achievable plasma concentrations of 8.8 nM (≙ 4.43 ng/mL) after multiple dose administration are much lower than those of hyperforin [16]. To conclude, no reduction in placental cell viability, increased apoptosis, and genotoxicity are expected to be clinically relevant in humans at the recommended doses of products containing St. John's wort extracts. Pregnant women should nevertheless preferentially be treated with preparations low in hyperforin.

In the *ex vivo* placental perfusion model using term placentae, however, only small amounts of hyperforin could be transported into the fetal circulation, and hypericin could not cross the placental barrier. Furthermore, neither compound affected metabolic (glucose consumption, lactate production) or functional (*β*-hCG and leptin production) parameters of the placenta, in addition to an inconspicuous global placental pathology. These observations speak in favor of a good safety of St. John's wort preparations for the fetus. Both, hyperforin (536.78 g/mol) and hypericin (504.44 g/mol) are relatively large molecules and are known for their physicochemical properties such as high insolubility and lipophilicity. The two constituents were therefore not suitable for use in some models such as the *in vitro* Transwell model as they could not cross artificial barriers (e.g., polycarbonate membrane inserts).

California poppy (*Eschscholzia californica*)

California poppy is not well known in Switzerland, which was seen in the results of our survey, as none of the pregnant women consumed this plant for the treatment of NMDs during pregnancy. Evidence for sedative, anxiolytic and spasmolytic effects of the plant are mainly based on many years of experience. Its effectiveness was only assessed either in combination with hawthorn and magnesium [17] or valerian extract [18], in open-label studies with few participants, or in patients with chronic pain [19]. Nevertheless, based on the available pharmaceutical products, the use of California poppies is widespread, especially in France and North America. In Switzerland, there is no product on the pharmaceutical market containing the extract of California poppy. Only one product is registered (Arkocaps® Escholtzia; Arko Diffusion SA) consisting of capsules containing the powdered drug (flowering herb) of California poppy [20].

Extracts of California poppy (≤ 30 μg/mL) and protopine (≤ 10 μM), an alkaloid of California poppy, showed no effects on *in vitro* safety parameters, as they did not exhibit cytotoxic or genotoxic potential, nor did they affect metabolic activity or differentiation of placental cells. Moreover, they were not able to significantly alter protein expression of BeWo b30 cells, suggesting that the use of California poppy extracts in pregnancy appears to be relatively safe, although plasma concentrations of protopine have not yet been published.

Ex vivo, protopine did not alter glucose consumption, lactate, and hormone production of the perfused placentae. In contrast, it was able to cross the placental barrier, and 20% of the initial concentration appeared in the fetal circulation after 6 hours. Whether this has an influence on the development of the unborn child remains unanswered. Given protopine's inconspicuous performance in all types of performed *in vitro* and *ex vivo* experiments, it is tempting to speculate that no harm is to be expected.

Valerian (*Valeriana officinalis*)

Valerian has a long tradition of use and is mainly known as a sleeping aid. Its efficacy in improving sleep quality has been demonstrated in several studies [21]. In pregnancy, the Swedish Birth Register reports common usage, and no adverse effect on pregnancy outcome has been reported [22]. *In vivo*, however, there are conflicting results, which is why the EMA does not recommend its use in pregnancy [23]. Other institutions, such as Embryotox [24] and SAPP [25], on the other hand, support the use of (non-alcoholic!) preparations in pregnancy. On the Swiss market there are some herbal preparations in solid or liquid form containing only valerian extract (Somnofor®, Sedonium®). More frequently, valerian occurs in combination with other plant extracts such as hop cones (Valverde® Schlaf, Redormin®, Hova®), lemon balm leaves (Dormiplant®) or both (Baldriparan®) [20].

In our survey, valerian was taken by pregnant women mainly in the last trimester for the treatment of sleep disorders and restlessness and was very effective with good to very good tolerability.

In vitro, valerian extract decreased the viability and increased the apoptosis rate of placental cells only at artificially high concentrations of 100 µg/mL. Under the influence of 30 µg/mL of valerian extract the proteome of BeWo b30 cells was not altered, as no biological changes in differential expression of proteins could be observed. Valerenic acid showed no adverse effects *in vitro* up to high concentrations of 30 µM, whereas valtrate, another constituent of valerian, exerted significant toxic effects, as cytotoxicity, apoptosis, and genotoxicity increased under the influence of ≥ 10 µM, and metabolic activity (glucose consumption, lactate production) was significantly decreased at levels as low as ≥ 1 µM valtrate. Based on these data, we think that valerenic acid is relatively safe in pregnancy, as no toxic effects were observed under expected maximum serum levels of 18.2 µM (≙ 2.8 µg/mL) after a single administration of 600 mg valerian (Sedonium, Lichtwer Pharma) via indwelling catheter in the arm vein [26]. Even lower plasma concentrations of valerenic acid are to be expected upon oral application. Regarding valtrate, there are no plasma concentrations available in literature, and effects should be interpreted with caution because valepotriates (e.g., valtrate, isovaltrate) are very unstable and easily degraded by heat, acids, or bases [27], meaning that they are not present in commercially available products containing valerian extract. In fact, we could not detect them when characterizing an extract of *V. officinalis* by HPLC-PDA-ESI-MS analysis (see Supporting Information in [28]). For this reason, we did not investigate the ability of valtrate to cross the placental barrier *in vitro* and *ex vivo*.

At the end of pregnancy, valerenic acid is able to cross the placental barrier unhindered, as same concentrations were detected in the maternal and fetal circulation after 4 hours of placental perfusion. A thorough histopathological examination revealed inconspicuous global placental pathology and unaltered metabolic and functional activity of the placentae after placental perfusions with valerenic acid. In the *in vitro* Transwell model, however, valerenic acid was unable to cross the placental cell layer. This speaks for the safety of valerenic acid in early pregnancy, where the unborn child is particularly susceptible to toxic environmental influences.

Lavender (*Lavandula angustifolia*)

Lavender is particularly known for its anxiolytic properties [29]. Although some studies demonstrate the clinical efficacy of lavender oil [30], safety data in pregnancy are largely lacking. To our knowledge, no study has investigated the safety of lavender oil preparations in pregnancy. Therefore, many institutions do not provide recommendations for its use in pregnancy.

Interestingly, our survey showed that lavender is one of the most frequently taken phytomedicine during pregnancy (16.2%, 56/345), which may be due to the increased number of women suffering from anxiety during vs. before pregnancy. Overall, its effectiveness was described as good to very good by almost half of the women (46.4%, 26/56), but bad by almost as many (35.7%, 20/56).

Neither lavender essential oil nor linalool, one of the two main components of the essential oil, had toxic effects on placental cells *in vitro*. Therefore, it was surprising that lavender oil exerted the greatest effect on protein expression, as a total of 24 proteins were significantly up- or down-regulated. However, considering the large diversity of proteins (3999 proteins in total), the impact was not significant. Moreover, no explicit signaling pathway was excessively involved.

Peak plasma concentrations of linalool are very low with 0.14 µM (≙ 22 ng/mL) and 0.85 µM (≙ 131 ng/mL) after single or multiple administrations of Lasea® (Dr. Willmar Schwabe GmbH & Co KG), respectively [31]. *In vitro*, we have included concentrations of up to 30 µM linalool, which did not cause any toxicity or impairment of placental cell function. Given that linalool is known to be a volatile substance, we measured its loss under our experimental *in vitro* experiments. In future experiments, it is advisable to carefully select the exposure time and even of the location of treated cells within cell plates. Due to the high volatility, we decided against investigating the placenta permeability of linalool.

Hops (*Humulus lupulus*)

Despite a long history of traditional use, clinical studies, and *in vivo* data on the safety of hop preparations in pregnancy are lacking. Institutions can therefore not provide any recommendations for use in pregnancy.

In our survey, women reported taking hops for the relief of stress, restlessness, or sleep disorders with a very good effectiveness. However, only a few women (2.3%, 8/343) indicated taking hops, which is significantly less than those reported taking valerian. A possible reason for this could be that women are unaware that most phytopharmaceuticals for sleep disorders and restlessness contain a combination of hops and valerian, and sometimes even other herbal extracts such as passionflower and lemon balm [20].

In vitro, hops extract had no harmful effect on placental cells at concentrations up to 30 µg/mL. Moreover, it could neither alter the metabolic activity of the cells nor inhibit or induce differentiation. The fact that the incubation with hops extract could not alter the protein expression also supports its safety.

New perspectives in herbal safety research in pregnancy

Our survey was conducted among pregnant women in the Canton of Zurich, where general interest in the use of phytotherapy is high and numerous herbal medicines are well known. In our experimental work, we focused on five phytomedicines that could be used as alternatives for the treatment of NMDs in pregnancy. We realized, however, that there are other medicinal plants that might be of interest, such as Bryophyllum/Goethe plant (*Kalanchoe pinnata*), cannabis (*Cannabis sativa*), kava kava (*Piper methysticum*), lemon balm (*Melissa officinalis*), or passionflower (*Passiflora incarnata*) [32]. This led us to the question, how should we address their safety in pregnancy in view of the lessons learned during the last four years of research.

To study other plants regarding their safety in pregnancy, testing of herbal extracts *in vitro* is certainly a good start to rule out cyto- and genotoxic effects in a physiological range. The experimental setup to investigate aspects, such as influence of herbal extracts on metabolic activity, placental cell differentiation or gene expression is relatively straightforward in its operation. Since we know very little about the pharmacological activities and target genes of herbal extracts and given that the metabolic pathways and phytochemistry are complex, the proteomics-based approach is highly recommended as it provides unbiased, high-throughput and quantitative results allowing the study of numerous proteins (of interest). Additionally, it provides valuable insights into toxicological metabolic pathways or enrichments of biological processes, molecular functions, cellular components, or protein classes.

To assess the safety of the fetus in case of exposure to herbal extracts, transplacental transport must be studied, favorably with both the *in vitro* Transwell in combination with the *ex vivo* human placental perfusion model. The former has a high throughput and can be employed for long exposure times up to several days, which are certainly advantages of the model. It is, however, a highly simplified model with absence of flow and hydrostatic pressure. However, the disadvantages of this method include the complexity of the experimental setup and a non-universal protocol for operation, leading to results that were non-reproducible in our laboratory setting. This, together with the use of only one cell line (BeWo b30) and lack of cellular complexity, constitutes a major drawback of the model. To be closer to reality, *in vitro* co-culture models should be used in the future, which include (primary human) placental endothelial cells and primary placental fibroblasts (to simulate villous stroma) together with BeWo b30 placental cells [33, 34]. The

interaction of different cell types, as well as the stiffness of the matrix or absence of blood flow exert an influence on the functionality of the tissue barrier; therefore placenta-on-a-chip models have gained application in research. These models have microchannels to mimic blood flow at the placental barrier [34, 35]. Further research should, apart from transplacental transport, also investigate whether phytomedicines affect the transport of important nutrients such as glucose, which is essential for fetal survival. Until now, it has not been used for the transport of phytochemicals, and we have noticed that this model is not suitable for some phytochemicals, such as hyperforin and hypericin, because their physicochemical properties do not allow them to pass artificial membranes. Additional models are therefore needed.

The use of the *ex vivo* model to study the safety of herbal medicines is questionable for several reasons: (i) only selected phytochemicals can be tested, not reflecting the complexity of the entire extract and therefore excluding synergistic effects of multiple phytochemicals, (ii) most molecules, and especially small (< 500 Da), lipophilic, and non-ionized ones [36], are highly likely to be placenta-permeable via passive diffusion, (iii) the model reflects only one stage (term) of pregnancy and cannot account for changes in placental structure and function, and (iv) it is a very complex and technically challenging model with a very low success rate of about 20% [37]. The throughput of the model is mainly reduced by availabilities (maternal health status, absent informed consent) and condition of placentas (such as damaged cotyledon surface, inappropriate fetal vessels, absence of fetal membrane). Despite its limitations, the *ex vivo* perfusion model uses structurally intact parts of the placenta, which best reflects the *in vivo* situation in humans, being often seen as the gold standard to investigate transplacental transport. In addition, the high (genetic) variability from placenta to placenta reflects the situation in the human population better compared to the genetically homogenous immortalized cell lines. The *ex vivo* placental perfusion approach therefore allows estimation of fetal exposure under physiological conditions, and direct effects on intact placental tissue can be studied, supporting the establishment of a realistic safety assessment of phytochemicals. Before performing an *ex vivo* placental perfusion, however, it is advisable to assess the stability and characterize placental metabolization of each phytochemical. As in our work, this can be accomplished by incubating the compound it in the various conditions (matrices) including placental homogenates. Experiments performed with hydrocortisone, a substance known to be metabolized in the placenta [38, 39], confirmed the feasibility of this approach (data not shown). Another proposed interest would be the investigation of the metabolism of phytochemicals by the gut microbiota upon oral administration and/or by the liver after intestinal absorption, and the characterization of possible metabolites using previously described *in vitro* models.

In addition to the *in vitro* and *ex vivo* models, the diversity of phytochemicals contained in extracts can also be tested *in silico*. Using complex pharmacokinetic models (PBPK), maternal-fetal drug exposure, transplacental permeability clearance and maternal/fetal tissue exposure can be calculated considering various parameters [40, 41]. This could constitute an initial screening instrument that reserves the time-consuming *in vitro* work for those phytochemicals most likely to cause safety issues.

Clinical translation of our findings – from bench to bedside

To increase the awareness of California poppy, such phytopharmaceuticals should be introduced to pharmacies and other healthcare facilities by on-site training sessions of sales representatives, which would consequently increase their use among patients in general (apart from pregnant women). Thus, it is quite possible that phytopharmaceuticals containing the extract of California poppy would establish themselves on the Swiss market, which in turn would certainly increase the interest in clinical studies.

The evidence-level on safety of treatments during pregnancy, and in particular on herbal medicines, should be improved. In obstetrics, evaluation of potential teratogenic effects of new and well-established drugs is essential, as are possible effects on intrauterine growth. The available experimental models can provide useful information on this to some extent but should be chosen carefully to answer the scientific question(s) in the best possible way. All models have certain limitations, and, above all, it is the distancing from reality that is most criticized.

Collection of data on the use of medication and monitoring of human births remains essential for the detection of birth defects. There are currently few or no reports of possible teratogenic effects of herbal medicines such as California poppy, lavender, and hops, which may be used to treat mild NMD. For this reason, institutions such as HMPC, Embryotox, and SAPP do not yet make recommendations for their clinical practice in pregnancy. For example, the Swedish National Medical Birth Register collects information from prenatal, delivery and neonatal care, providing valuable information such as medical drug use during pregnancy, diagnoses before and during pregnancy, mode of delivery, fetal presentation, and equal importantly outcome data such as birth weight, body length and head circumference of the infant and fetal diagnoses including malformations [42]. In this context, personal data may be processed for research purposes, which is also necessary for reasons of substantial public interest [43].

Most of the herbal medicines studied in this work, such as St. John's wort, valerian, hops, and lavender, are already being used in pregnancy. Therefore, it is particularly important in the future that safety recommendations can be made based on evidence-based information. To obtain information on medication use and pregnancy outcome, there are several approaches: (i) locally, with a digital application (eMutterPass, University Hospital Zurich) which contains all relevant medical information of a pregnancy (e.g., personal history, prescribed drugs, ultrasound data, fetal growth chart, scan images). A survey showed that women would be willing to provide detailed information on their (non-prescribed) medication consumption interactively by taking pictures of drug packaging, filling in medication dosages or submitting assessments of perceived drug effectiveness. In addition, most women do not seem to be concerned about data security and confidentiality [44]: (ii) nationally, through the introduction of the Electronic Patient Record (EPR) in Spring 2020 where health professionals such as physicians and pharmacists can collect treatment-related documents of patients (including pregnant women) in Switzerland. The EPR also offers the possibility of providing an overview of medication use (also during pregnancy), including documentation of herbal medicines use which are available OTC [45]. However, it is not yet clear to what extent access rights and data protection laws permit data analysis for research purposes. Moreover, the way the data is stored (as PDF files) does not allow the direct analysis of the data.

To summarize, our studies with St. John's wort, California poppy, valerian, lavender, and hops, contribute to a better understanding of their toxic potential for the treatment of NMDs under the very delicate circumstances of pregnancy. All tested extracts were harmless in our *in vitro* assays, whereas some of the individual phytochemicals – *nota bene* at very high, likely not clinically relevant concentrations – were rather conspicuous. Assessing the safety of herbal extracts, however, has many pitfalls and is a permanent challenge, since they are multicomponent mixtures, and the variety of phytochemicals is striking. Future research efforts should continue to elucidate the safety of herbal extracts using the above-mentioned approaches, preferably with the discussed improvements. Our long-term goal should be to provide pregnant women with safe alternatives for the treatment of their conditions and symptoms and to alleviate their fear of adverse effects, associated pregnancy complications and fetal harm.

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