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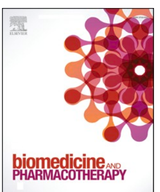

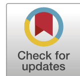

Alkaloids in commercial preparations of California poppy – Quantification, intestinal permeability and microbiota interactions

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ABSTRACT

California poppy products are commonly used for the treatment of nervousness, anxiety and sleeping disorders. Pharmacologically relevant constituents include the main alkaloids californidine, escholtzine and protopine. However, only limited information is available about the alkaloid content in commercial preparations and their intestinal absorption. Moreover, a possible metabolization of these alkaloids by the gut microbiota, and their impact on microbial activity and viability have not been investigated. Californidine, escholtzine and protopine were quantified by UHPLC-MS/MS in eight commercial California poppy products. The intestinal permeability of alkaloids was studied in Caco-2 cell as a model for absorption in the small intestine. The gut microbial biotransformation was explored in artificial gut microbiota from the in vitro PolyFermS model. In addition, the impact of these alkaloids and a California poppy extract on the microbial production of short-chain fatty acids (SCFAs) and the viability of microbiota was investigated. Contents of californidine, escholtzine and protopine in California poppy products were in the ranges of 0.13–2.55, 0.05–0.63 and 0.008–0.200 mg/g, respectively. In the Caco-2 cell model, californidine was low-to-moderately permeable while escholtzine and protopine were highly permeable. An active transport process was potentially involved in the transfer of the three alkaloids. The three compounds were not metabolized by the artificial gut microbiota over 24 h. Neither the California poppy extract nor the alkaloids markedly impacted microbial SCFA production and bacterial viability.

1. Introduction

Phytomedicines containing California poppy (*Eschscholzia californica*, Cham., Papaveraceae) are widely used for the treatment of nervousness, anxiety and sleeping disorders [1]. Reported pharmacological activities include modulation of γ-aminobutyric acid (GABA) receptor activity [2], serotonin receptor binding [3], and modulation of catecholamine metabolism [1,4]. The sedative, anxiolytic and analgesic effects of California poppy are associated, at least in part, with the alkaloids present in the plant. These include californidine, escholtzine and protopine (Fig. 1), with californidine as the most abundant, followed by escholtzine [1]. Previous studies have shown that these compounds could be involved in herb-drug interactions. Escholtzine was reported to increase CYP3A4 and CYP1A2 expression in HepG2 cells via activation of the pregnane X receptor (PXR), while californidine, escholtzine and protopine were found to inhibit the activity of certain cytochrome P450 enzymes (CYPs) using human liver microsomes and P-glycoprotein (P-gp) in MDCK-II cells [5].

The gut microbiota is highly diverse and ensures key functions in human health by regulating host immunity, modulating the intestinal barrier, protecting against pathogenic microbials, or providing energy to the host by metabolizing undigested food such as dietary fibers [6]. Dietary fibers and plant-based polysaccharides are precursors of short-chain fatty acids (SCFAs) [7], which serve as an energy source for intestinal cells, as signaling molecules, modulators of lipid metabolism, and as regulators of the intestinal immunity [8]. Besides these essential functions for human health, the gut microbiota has significant metabolic activity [9]. There is a growing number of studies identifying microbial biotransformation of ingested compounds (xenobiotics) by human fecal microbiota, or by single bacteria and synthetic bacterial consortia [10–13]. As a relevant in vitro system to study the metabolic activity and viability of gut microbiota, the artificial gut fermentation model

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1 2 3

Fig. 1. Chemical structures of californidine (**1**), escholtzine (**2**) and protopine (**3**).

Polyfermentor Intestinal Model (PolyFermS) enables reproducible and stable cultivation of colon bacterial communities derived from a single human fecal donor [14]. The model has been previously used to study the gut microbiota interactions with constituents from St. John's wort and valerian [15].

Information on the alkaloidal content in commercial products of California poppy remains limited. Furthermore, the intestinal absorption and a possible metabolization by the gut microbiota of these alkaloids have not been investigated up to now. The impact of these alkaloids and of a California poppy extract on the viability and metabolic activity of gut microbiota has also not been explored. In this study, californidine, escholtzine and protopine were quantified in eight California poppy products sold as phytomedicines or food supplements. The bidirectional transport of californidine, escholtzine and protopine in the Caco-2 cell model, and their stability in artificial gut microbiota from the in vitro PolyFermS model were investigated. In addition, the impact of these alkaloids and of a California poppy extract on bacterial viability and microbial SCFAs production was studied.

2. Materials and methods

2.1. Cell lines, chemicals and biochemicals

The Caco-2 cell line was kindly provided by Prof. Per Artursson, Uppsala University, Sweden. Dulbecco's Modified Eagle's Medium (DMEM, with high glucose, L-glutamine, phenol red, without sodium pyruvate), Dulbecco's phosphate-buffered saline (DPBS, without calcium/magnesium, without phenol red), fetal bovine serum (FBS), MEM non-essential amino acids solution (NEAA, without L-glutamine), penicillin-streptomicin (PEST, 10,000 U/mL), and trypsin (2.5%, without phenol red) were purchased from Gibco (Paisley, UK). Hank's balanced salt solution (HBSS, without phenol red), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 4-morpholineethanesulfonic acid monohydrate (MES), sodium bicarbonate ($NaHCO_3$), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), cellobiose, soluble potato starch, amicase, meat extract, mucin from porcine stomach type II, arabinogalactan from larch wood, Tween 80, hemin, $NaHCO_3$, KCl, $MgSO_4$, $CaCl_2 2H_2O$, $MnCl_2$, $FeSO_4 7H_2O$, $ZnSO_4 7H_2O$, 4-aminobenzoic acid (PABA), nicotinic acid, biotine, folic acid, cyanocobalamin, thiamine, riboflavin, phylloquinone, menadione, and pantothenate were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Xylan from oat spelts, and bacto tryptone were from Chemie Brunschwig (Basel, Switzerland). Inulin was provided by Cosucra (Warcoin, Belgium), and yeast extract by Lesaffre (Marcq-en-Barœul, France). Bile salts were purchased from Thermo Fischer Diagnostics (Pratteln, Switzerland). KH_2PO_4, NaCl, pyridoxine-HCl (Vit. B6), folic acid and menadione were obtained from VWR International (Dietikon, Switzerland). Ethylenediaminetetraacetic acid (EDTA, 0.2% in PBS, without calcium/magnesium) was purchased from MP biomedicals (Santa Ana, CA, USA). Culture flasks (75 cm^2), bottle top filters (pore size 0.22 µm), and 12-well Costar® plates, 12-well Transwell® plates (with 0.4 µm pore polycarbonate membrane inserts) were purchased from Corning Inc. (Corning, NY, USA). Sodium fluorescein salt, atenolol, propranolol HCl, verapamil HCl were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Californidine perchlorate (CAS n° 17939–31–0) was obtained from Phytolab (Vestenbergsgreuth, Germany), and protopine (CAS n° 130–86–9) from Extrasynthese (Genay, France). Escholtzine (CAS n° 4040–75–9) was isolated from *E. californica* aerial parts (See Supplementary Material).

Acetonitrile (MeCN) and acetone were from Supelco (Bellefonte, PA, USA). Methanol (MeOH), isopropanol (i-PrOH) and ethanol (EtOH) were obtained from Macron Fine Chemicals (Radnor, PA, USA). *n*-Butanol (BuOH) and ethyl acetate (EtOAc) were purchased from Scharlau (Sentmenat, Spain). All solvents were of UHPLC grade. HPLC grade water was obtained from a Milli-Q integral water purification system (Millipore Merck, Darmstadt, Germany). Materials for flow cytometry were purchased from Beckman Coulter International (Nyon, Switzerland), except for stains SYBR Green I and propidium iodide which were from Life Technologies Europe (Zug, Switzerland).

2.2. Plant material and extraction

California poppy commercial preparations included different galenic forms. Three preparations were capsules or tablets containing dry flowering parts of the plant: Arkogélules® Escholtzia (Arkopharma, Carros, France), Herbes & Plantes Escholtzia Bio (Herbes et Plantes, Magescq, France), and Nature & Plantes Escholtzia (Planète au naturel, Magescq, France). Three preparations were fluid extracts of flowering aerial parts: Sommeil Pavot jaune de Californie Bio (Weleda, Arlesheim, Switzerland), Sommeil Pavot jaune de Californie (Boiron, Messimy, France), and Escolzia (Naturalma, Bologna, Italy). Phytostandard Eschscholtzia Valériane consisted of tablets containing extracts of valerian roots and California poppy flowering aerial parts (PiLeJe laboratoire, Paris, France). Elusanes Eschscholtzia were capsules containing an extract of flowering aerial parts (Pierre Fabre, Castres, France). Manufacturer's information on the composition of all preparations is provided in Table S1. Capsules or tablets of flowering parts were extracted with 100% MeOH utilizing pressurized liquid extraction (PLE) with a Dionex ASE 200 instrument (Sunnyvale, CA, USA). Three cycles of extraction of 5 min each at a temperature of 70 °C and a pressure of 120 bar were applied. The extracts were evaporated under reduced pressure and lyophilized.

The plant material used for the batch fermentation experiments and for the isolation of escholtzine was of Ph. Eur. grade and was purchased from Galke (Bad Grund, Germany). A voucher specimen (No 1234) has been deposited at the Division of Pharmaceutical Biology, University of Basel. The material was extracted by PLE with 70% EtOH. Three extraction cycles of 5 min were performed at a temperature of 70 °C and a pressure of 120 bar. The herbal extract has been previously characterized [16].

2.3. Caco-2 cell model and permeability experiments

2.3.1. Cell culture and permeability experiments

Cell culture and assay conditions were as previously published [15]. Briefly, cells were grown in 75 cm^2 culture flasks using DMEM (supplemented with 10% FBS, 1% NEAA, 1% PEST) in a humidified atmosphere (95%) with 10% CO_2, at a temperature of 37 °C. When confluence of 90–95% was reached, cells were subcultured by dissociation with trypsination solution (0.25% trypsin, 0.2% EDTA). To

prepare monolayers for permeability experiments, cells were seeded at a density of 4.5×10^5 cells/cm^2 and cultivated on Transwell® inserts for 22–28 days. The medium was changed three times a week. The seeding on inserts was performed with cells corresponding to passage number 94–108.

Permeability across Caco-2 monolayers was studied in both directions, from apical to basolateral (AB), and from basolateral to apical (BA) at a final concentration of 10 μM of test compounds in the donor chamber and with 1% DMSO as co-solvent, over 60 min with 15 min sampling time intervals. Sampling volume was 0.6 mL or 0.2 mL from the basolateral chamber or apical chamber, respectively, and the sampling volume was replaced by fresh transport buffer. To simulate physiological conditions found in the human small intestine, transport buffers were adjusted to pH 6.5 in the apical chamber (10 mM MES, 4.2 mM $NaHCO_3$ in HBSS), and to pH 7.4 in the basolateral chamber (25 mM HEPES, 4.2 mM $NaHCO_3$ in HBSS). Permeability experiments were performed in triplicate. Collected samples were precipitated with MeCN and stored at −80 °C until UHPLC-MS/MS analysis. Cumulative fraction curves for each experiment of all compounds are shown in Figs. S1–S5.

2.3.2. Cell lysis

Following permeability experiments, cell monolayers were washed quickly with HBSS/HEPES buffer at pH 7.4 and lysed with MeCN for 30 min. Cell lysis experiments were performed in duplicate. Samples were stored at −80 °C until UHPLC-MS/MS analysis.

2.3.3. Control of Caco-2 cell monolayer integrity

Monolayer integrity was controlled using transepithelial electrical resistance (TEER) and the paracellular leakage marker fluorescein as described before [15]. Only monolayers with TEER values above 200 Ω cm^2 were used (Table S2) and a passage below 1% of fluorescein per hour was used as an indicator of monolayer integrity (Table S2) [17]. As an additional control, atenolol and propranolol were included as references for low-to-moderately and highly permeable drugs, respectively.

2.3.4. Experiments on cell-free inserts

Control experiments with cell-free inserts were performed to assess the passage of the compounds in absence of cells. As for the permeability experiments, study compounds were added to the donor chamber and incubated for 1 h at 37 °C under shaking (450 rpm). At the end of the experiment, aliquots were taken from donor and receiver chambers, and stored at −80 °C until analysis by UHPLC-MS/MS.

2.3.5. Determination of apparent permeability coefficients, efflux ratio and recoveries

Apparent permeability coefficients (P_{app}), efflux ratio and recovery values (recovery considering A and B chambers (Re_{AB}), and recovery considering A, B and the cell fraction (Re_{ABC})) were calculated as previously reported [15]. Equations are given in the supplementary material.

2.4. In vitro gut microbiota experiments

2.4.1. Anaerobic batch fermentation experiments with artificial gut microbiota and California poppy extracts or compounds

To assess the gut microbial biotransformation capacity, anaerobic batch fermentations with artificial gut microbiota and California poppy extract and compounds were performed using the same setup as previously reported for valerian and St. John's wort compounds [15].

The two artificial human gut microbiota used in this study were derived from independent stable PolyFermS bioreactors that were both inoculated with immobilized fecal microbiota from two healthy female individuals (age 25–35) and operated as previously described [15]. The PolyFermS system is designed and operated to mimic the proximal colon conditions and allows to continuously cultivate the proximal colon microbiota akin to donor profile [18,19]. For each anaerobic batch experiment the artificial human colon microbiota from a single stabilized PolyFermS bioreactor were completely harvested under anaerobic conditions (10% CO_2, 5% H_2 and 85% N_2) in an anaerobic tent (Coy Laboratories, MA, USA).

PolyFermS microbiota were incubated with herbal compounds to monitor the colon microbial biotransformation potential and impact on fermentation metabolites and bacterial viability as described before [15]. In short, fresh and metabolically highly active PolyFermS colon microbiota effluent was supplemented with nutritive medium (MacFarlane medium) at a ratio of 7:3, and adjusted to pH 6.5. The high microbiota to medium ratio was chosen to enable the evaluation of the microbial biotransformation of the compounds and impact of the compounds on microbial viability under limited growth conditions. California poppy extract and compounds (californidine, escholtzine, protopine) were dissolved in DMSO and added to 10 mL microbiota mixture, at a final concentration of 500 μg/mL or 30 μg/mL, respectively. The final DMSO content was set to 0.2% (v/v) for single compounds and to 0.5% (v/v) for the plant extract, based on previous experiments showing low impact on SCFA production at these concentrations [15]. Microbiota-nutrient-compound mixtures were prepared under anaerobic conditions and filled into sterile serum flasks that were closed with sterile butyl rubber septa. Incubations were performed in the dark, at 37 °C, and shaking (100 rpm) for 24 h, and under strict anaerobic conditions. 1 mL sample was withdrawn before (T0h) and after 24 h (T24h) of incubation and stored at −80 °C until extraction and UHPLC-MS/MS analysis. For each experiment, DMSO controls with 0%, 0.2% and 0.5% DMSO (v/v) and 0.001% sodium perchlorate (w/v) were included. A 1 mL sample of microbial content was collected at the end of the experiment for SCFAs and bacterial cell quantification. All incubation experiments were performed in triplicate with the two artificial microbiota. Additionally, study compounds were incubated under same conditions with microbial-free (sterilization through 0.20 μm filter) incubation mixture to assess degradation in abiotic conditions.

2.4.2. Fermentation metabolite quantification

To evaluate the impact of California poppy compounds or extract exposure on microbial metabolism, the fermentation metabolites acetate, propionate, butyrate, succinate, valerate, isovalerate and isobutyrate were quantified in collected fermentation samples as described before [15]. In short, supernatant of 1 mL fermentation samples (13′000 cfm for 10 min at 4 °C) was filtered (0.2 μm nylon filter) and analyzed by HPLC (LaChrom, Merck-Hitachi, Germany, or Accela, Thermo Fisher Scientific, Reinach, Switzerland) equipped with a Security Guard Cartridge Carbo-H (4 ×3.0 mm) and a Rezex ROA-Organic Acid H+ (300 ×7.8 mm) column (Phenomenex, Basel, Switzerland) and a refractive index detector (Thermo Fisher Scientific). The injection volume was 20 μL (Accela HPLC) or 40 μL (LaChrom HPLC). The mobile phase used was 10 mM H_2SO_4 at a flow rate of 0.4 mL/min at 40 °C for 60 min per sample under isocratic conditions. The metabolites were quantified by external standard calibration.

2.4.3. Bacterial cell quantification

Bacterial flow cytometry was used to determine the total viable and dead bacterial cell counts of microbiota incubated with or without California poppy compounds or extract. The assay is based on a bacterial cell staining with a live/dead staining that consists of two DNA-binding fluorescent stains: SYBR® Green I and propidium iodide (PI). The former penetrates all cells and results in a green fluorescence, the latter penetrates only cells with a damaged cell membrane resulting in red fluorescence. After staining, the amount of cells with intact (viable) and permeable (dead) membrane were determined in each sample with a flow cytometer (Cytomics FC 500, Beckman Coulter) following the same protocol as described before [15]. The collected cell concentrations were exported to Microsoft Excel and converted to cell count/mL, taking into

account the sample dilution factor.

2.4.4. Microbiota sample extractions

Microbiota samples were thawed and directly processed by sequential liquid-liquid extractions with EtOAc and *n*-BuOH. Aliquots of 1 mL were extracted with 1 mL EtOAc and centrifuged for 10 min at 20 °C and 3000 rpm (Centrifuge 5810 R, Eppendorf, Hamburg, Germany). Then, the supernatant was collected and further extracted with 1 mL EtOAc, followed by 1 mL *n*-BuOH. The extracts were combined, evaporated to dryness using a rotary evaporator (Buchi, Flawil, Switzerland) and stored at −80 °C until analysis by UHPLC-MS/MS.

2.5. UHPLC-MS/MS analysis

2.5.1. Instrumentation and chromatographic conditions

California poppy, Caco-2 and microbiota samples were analyzed by ultrahigh performance liquid chromatography (UHPLC) coupled to electrospray ionization (ESI) tandem mass spectrometry (MS/MS) in multiple reaction monitoring (MRM) mode and with verapamil as internal standard (IS).

Californidine, escholtzine, protopine, atenolol and propranolol were analyzed on a 1290 Infinity UHPLC system consisting of a binary pump, an autosampler and a thermostatted column compartment coupled to a 6460 triple quadrupole mass spectrometer (all Agilent, Waldbronn, Germany). MS parameters (MRM transitions, cone voltage and collision energy) for MRM were optimized using the Agilent MassHunter program Optimizer and are presented in Table S3. UHPLC parameters of all study compounds such as gradient, flow rate, run time, eluents, column, injection volume, column temperature and autosampler temperature are listed in Table S4.

2.5.2. Sample preparation prior to UHPLC-MS/MS of California poppy samples

Dry extracts were reconstituted in appropriate volume of DMSO to reach a final concentration of 10 mg/mL, sonicated for 15 min, and centrifuged for 20 min (3500 rpm, 25 °C). 20 μL of supernatant were collected and further diluted with DMSO. The liquid preparations of California poppy were diluted with DMSO.

50 μL of analyte (californidine, escholtzine or protopine) diluted in DMSO were precipitated with 200 μL MeOH containing the IS at a concentration of 800 ng/mL, and then centrifuged for 20 min (12,700 rpm, 10 °C). 100 μL supernatant was collected and analysed by UHPLC/MS-MS.

2.5.3. Sample preparation prior to UHPLC-MS/MS analysis of Caco-2 samples

2.5.3.1. Californidine. To 50 μL of analyte in a mixture of HBSS and MeCN (1:1) were added 150 μL of ice-cold MeOH (containing the IS at a concentration of 1000 ng/mL). The samples were mixed for 10 min at room temperature on an Eppendorf MixMate (Hamburg, Germany), and centrifuged for 20 min (12,700 rpm, 10 °C). 100 μL supernatant were collected in 96-deepwell plate (96-DPW, Biotage, Uppsala, Sweden) and analysed by UHPLC/MS-MS.

2.5.3.2. Escholtzine. To 200 μL of analyte in a mixture of HBSS and MeCN (1:1) were added 900 μL of ice-cold MeCN (containing the IS at a concentration of 100 ng/mL). The samples were mixed for 10 min at room temperature, and centrifuged for 20 min (12,700 rpm, 10 °C). 750 μL supernatant were collected and transferred into a 96-deepwell plate (96-DPW, Biotage) and dried under nitrogen gas flow (Evaporex EVX-96, Apricot Designs, Covina, CA, USA). Samples were redissolved with 200 μL of a mixture of water and MeCN (65:35), both containing 0.1% of formic acid, followed by 30 min of shaking on an Eppendorf MixMate.

2.5.3.3. Protopine. The UHPLC-MS/MS method used for protopine quantification in Caco-2 samples has been previously reported [20].

To 200 μL of analyte in a mixture of HBSS and MeCN (1:1) were added 200 μL of 6% BSA in water, 100 μL of MeOH (containing the IS at a concentration of 1000 ng/mL) and 800 μL of ice-cold MeCN. The samples were mixed for 10 min at room temperature on an Eppendorf MixMate, and centrifuged at 10 °C for 20 min at 12,700 rpm. An aliquot of 750 μL supernatant was collected and transferred into a 96-deepwell plate (96-DPW, Biotage) and dried under nitrogen gas flow (Evaporex EVX-96). Samples were redissolved with 200 μL of a mixture of water and MeCN (65:35), both containing 0.1% of formic acid, followed by 30 min of shaking on an Eppendorf MixMate.

2.5.3.4. Atenolol and propranolol. To 200 μL of analyte in a mixture of HBSS and MeCN (1:1) were added 100 μL of 6% BSA in water and 900 μL of ice-cold MeCN (containing the IS at a concentration of 100 ng/mL). The samples were mixed for 10 min at room temperature on an Eppendorf MixMate, and centrifuged at 10 °C for 30 min at 3500 rpm. An aliquot of supernatant was collected (800 μL for atenolol, 300 μL for propranolol) and transferred into a 96-deepwell plate (96-DPW, Biotage) and dried under nitrogen gas flow (Evaporex EVX-96). Samples were redissolved with 100 μL (atenolol) or 200 μL (propranolol) of a mixture of water and MeCN (65:35), both containing 0.1% of formic acid, followed by 30 min of shaking on an Eppendorf MixMate [15].

2.5.4. Sample preparation prior to UHPLC-MS/MS of microbiota samples

Microbiota samples were extracted as mentioned above (Section 2.4.4). Microbiota dry samples T0h and T24h were reconstituted with DMSO (containing the corresponding internal standard at a concentration of 500 ng/mL) and analysed by UHPLC-MS/MS. Chromatographic and MS/MS conditions were as for California poppy and Caco-2 sample analysis.

2.5.5. UHPLC-MS/MS quantification methods and acceptance criteria

UHPLC-MS/MS methods for absolute quantification of study compounds in analytical samples from California poppy products and Caco-2 experiments consisted in the injection of 2 sets of 7 calibrator samples validated with 2 sets of 3 quality control (QC) samples from the low, middle and high level of the calibration curve. Calibrators, QC and analytical samples were processed with the same sample preparation protocol, and prepared fresh prior to UHPLC-MS/MS analysis. To be accepted, a bioanalytical run was required to have a coefficient of determination (R^2) higher than 0.96 with at least 75% of all calibrators valid. Additionally, for the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ), at most one value could be excluded. Furthermore, between bioanalytical runs, the imprecision (CV %) had to be lower than 15% (20% at the LLOQ), and the inaccuracy (RE %) had to be within ± 15% (± 20% at the LLOQ). The above mentioned criteria were in accordance with requirements of the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for bioanalytical methods [21,22]. For California poppy extract samples analysis, calibration range was from 2.5 to 1000 ng/mL for californidine, from 2.5 to 250 ng/mL for escholtzine, and from 2.5 to 500 ng/mL for protopine. For analysis of Caco-2 samples, the calibration range was from 0.1 to 125 ng/mL for californidine, from 10 to 1000 ng/mL for escholtzine, and from 2.5 to 500 ng/mL for protopine. For the control compounds propranolol and atenolol, the range was from 10 to 2000 ng/mL. For very low concentrated samples of atenolol, an additional UHPLC-MS/MS method was developed in a lower range of 0.05–10 ng/mL [15]. Calibration curves are provided in Figs. S6–S14, and curve parameters, calibrator and QC samples are in Tables S5–S22. Additionally, carry-over values were determined to not exceed 20% for the analyte, and 5% for the internal standard (Tables S23–S24).

Table 1
Content of californidine, escholtzine and protopine in commercial products of flowering aerial parts of California poppy. Data are reported as mg/g (for solid preparations), or mg/mL (for liquid preparations) ± SD.

Product n°	Galenic form	Californidine	Escholtzine	Protopine
1	Dry powder	2.00 ± 0.08	0.39 ± 0.01	0.17 ± 0.01
2	Dry powder	1.78 ± 0.05	0.63 ± 0.02	0.145 ± 0.004
3	Dry powder	1.57 ± 0.04	0.42 ± 0.01	0.104 ± 0.004
4	Dry extract	2.55 ± 0.02	0.38 ± 0.01	0.20 ± 0.01
5	Dry extract	0.76 ± 0.12	0.39 ± 0.02	0.11 ± 0.01
6	Fluid extract	0.153 ± 0.005	0.085 ± 0.001	0.058 ± 0.002
7	Fluid extract	0.152 ± 0.004	0.054 ± 0.001	0.0081 ± 0.0003
8	Fluid extract	0.131 ± 0.004	0.084 ± 0.002	0.015 ± 0.001

2.6. Data acquisition and statistical analysis

UHPLC-MS/MS data were acquired and processed using Agilent MassHunter version 10.0, or Waters MassLynx V4.1 software. Statistical analysis was performed and graphs drawn with GraphPad Prism 9.3.1.

3. Results and discussion

3.1. Quantification of californidine, escholtzine and protopine in commercial preparations

Highly sensitive and selective UHPLC-MS/MS methods in the MRM detection mode were developed for the quantification of californidine, escholtzine and protopine in commercial preparations (Section 2.5.5). For each method, the imprecision (expressed as CV%) and inaccuracy (expressed as CV%) of calibration levels and QC levels were both below 15% (20% at the LLOQ) (Tables S5–S10), showing that the methods were precise and accurate [21,22]. Also, carry-over values were below 20% for the analyte and below 5% for the IS, showing that carry-over had no impact on quantification (Table S23). Therefore, the methods were reliable for the quantification of the alkaloids in commercial formulations of California poppy.

The methods were applied for the determination of californidine, escholtzine and protopine in eight commercial preparations of flowering aerial parts of California poppy containing dry herbal powder (preparations 1–3), dry extract (preparations 4 and 5), or fluid extract (preparations 6–8) as the active ingredient (Tables 1, S1). All preparations showed a similar alkaloid pattern, but with variations in absolute amounts of compounds. Californidine was the most abundant, followed by escholtzine and protopine. The contents in dry powders (preparations 1–3) were comparable, with contents in californidine, escholtzine and protopine of 1.57–2.00, 0.39–0.63, and 0.10–0.17 mg/g, respectively. In contrast, important differences were noticed between dry extracts, with respective amounts of californidine, escholtzine and protopine of 2.55, 0.38 and 0.20 mg/g in preparation 4, and of 0.76, 0.39 and 0.11 mg/g in preparation 5. In fluid extracts (preparations 6–8), contents of californidine, escholtzine and protopine were in the ranges of 0.131–0.153, 0.054–0.085, and 0.008–0.058 mg/g, respectively. The concentration of protopine, in particular, differed strongly between the products (Table 1). The differences observed in the extract-containing products are likely due, at least in part, to differing extraction procedures.

There have been a few studies on the contents of these three alkaloids in commercially available preparations of California poppy. The contents determined by HPLC-UV in two herbal powders and one solid extract were in a similar range for californidine and protopine, while the contents of escholtzine were up to ten times higher compared to the values found in preparations 1–5 [23]. The contents of californidine and escholtzine determined by capillary electrophoresis in a commercial tincture of California poppy were comparable to those in preparations 6–8, whereas the content of the major alkaloid protopine was comparable to that of preparation 7 [24].

Based on our data and the dosage recommendations of the manufacturers, the maximum amounts of californidine, escholtzine and protopine ingested per day were calculated to range between 0.16 and 2.97 mg/day (californidine), 0.10–1.11 mg/day (escholtzine), and 0.02–0.31 mg/day (protopine) (Fig. 2). Thus, considerable differences in the daily intake of californidine, escholtzine and protopine are expected for patients using different California poppy products.

3.2. Permeability across Caco-2 monolayers

The intestinal absorption of californidine, escholtzine and protopine was assessed by means of permeability across Caco-2 cell monolayers. The apparent permeability coefficients (P_{app}) from apical to basolateral (AB), and from basolateral to apical (BA) for each of the compounds were determined. Atenolol and propranolol were used as controls as previously described [15] and results were within the reference range (Table 2).

Californidine showed a mean P_{app}AB of 0.58×10^{-6} cm/s, a mean P_{app}BA of 4.93×10^{-6} cm/s, and a calculated ER of 8.6. Re_{AB} values in AB direction and BA direction were 90.3% and 114%, respectively.

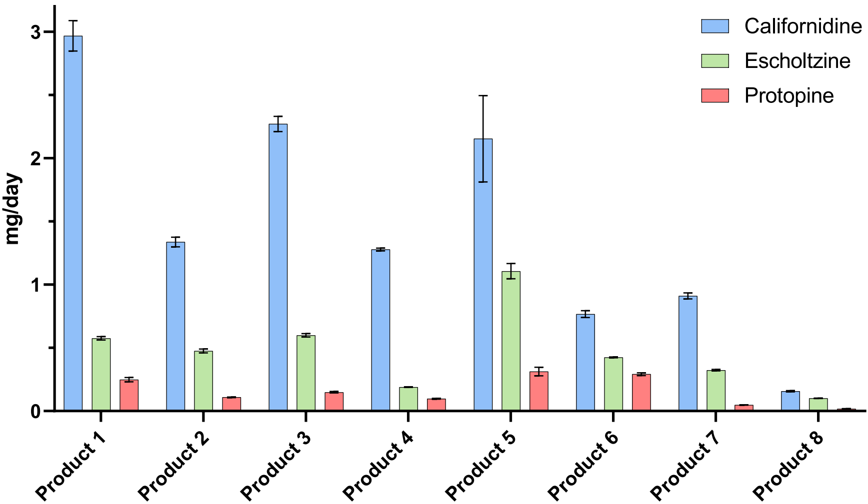

Fig. 2. Maximum daily intake of californidine, escholtzine and protopine for commercial products 1–8. Calculations are based on contents (Table 1) and dosage recommendations provided by the manufacturer (Table S1). Data are reported in mg / day ± SD.

Table 2
Permeability data of californidine, escholtzine, protopine, atenolol, and propranolol across Caco-2 monolayers. Data are presented as mean ± SD. (n = 3). Cumulative fraction curves for each individual monolayer are shown in Figs. S1–S5.

Compounds	AB direction			BA direction			
	$P_{app}AB$ ($\times 10^{-6}$ cm/s)	Re_{AB}[a] (%)	Re_{ABC}[b] (%)	$P_{app}BA$ ($\times 10^{-6}$ cm/s)	Re_{AB} (%)	Re_{ABC} (%)	ER[c]
Californidine	0.58 ± 0.13	90.3 ± 2.7	98.2	4.93 ± 0.89	114 ± 60	115	8.6
Escholtzine	82.5 ± 4.1	17.7 ± 4.4	37.6	175 ± 5	42.8 ± 11.1	53.6	2.1
Protopine	39.2 ± 2.4	36.7 ± 1.5	38.8	144 ± 4	11.9 ± 5.0	13.0	3.7
Atenolol[d]	0.11 ± 0.03	88.7 ± 6.3	92.8	0.14 ± 0.02	118 ± 22	118	1.3
Propranolol[d]	53.4 ± 5.3	39.5 ± 1.1	58.1	121 ± 4	61.8 ± 5.2	69.8	2.3

[a] Recovery considering apical and basolateral compartments
[b] Recovery considering apical and basolateral compartments, and cell fraction
[c] Efflux ratio
[d] Values from our previous study [15].

Re_{ABC} values in AB direction and BA direction were 98.2% and 115%, respectively (Table 2). Permeability experiments across empty inserts revealed that the passage of californidine was not restricted (Fig. S15). The $P_{app}AB$ classified californidine as a low-to-moderately permeable compound. Californidine is a quaternary ammonium compound carrying a permanent charge, which is expected to restraint its permeability across epithelial barriers [25]. The calculated ER of 8.6 suggests that an active efflux process could be involved in the transport of californidine [26].

For escholzine, the mean $P_{app}AB$ was 82.5×10^{-6} cm/s, the mean $P_{app}BA$ 175×10^{-6} cm/s, and the ER 2.1. In AB direction, the Re_{AB} was 17.7%, and Re_{ABC} was 37.6%. In BA direction, the Re_{AB} was 42.8%, and Re_{ABC} was 53.6% (Table 2). Protopine showed a mean $P_{app}AB$ of 39.2×10^{-6} cm/s and a mean $P_{app}BA$ of 144×10^{-6} cm/s, with a calculated ER of 3.7. Re_{AB} values in AB direction and BA direction were of 36.7% and of 11.9%, respectively. Re_{ABC} values in AB direction and BA direction were of 38.8% and of 13.0%, respectively (Table 2). Both alkaloids were classified as highly permeable compounds [26]. Data for protopine were consistent with previously reported pharmacokinetic data in various animal models [27]. The obtained ER values suggest that an active efflux transport could be involved in the transfer of both compounds. Further experiments at lower compound concentration to reduce possible transport saturation and/or with specific carrier-protein inhibitors would be needed to confirm the involvement of active efflux processes. The low recovery values (Table 2) suggest that escholtzine and protopine may be metabolized given that solubility issues could be ruled out (Fig. S15). In Caco-2 cells, levels of phase I metabolizing enzymes are known to be low, while phase II enzymes such as UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs) and glutathione S-transferases (GSTs) are highly expressed [28] which could possibly have contributed to the low recovery. In agreement with this, protopine glucuronide conjugates have been detected in rat urine samples [27].

As a limitation inherent to the Caco-2 cell model the P_{app} values are considered to be slightly underestimated for values of recovery below 80%, and should, therefore, be seen as a qualitative readout [26]. This was the case for escholtzine and protopine, as well as for the control propranolol.

3.3. Stability of compounds during in vitro gut microbiota fermentation

Incubation experiments in artificial microbiota were performed to assess a possible biotransformation of californidine, escholtzine and protopine by human gut microbiota. Two microbiota derived from two different healthy adult female fecal donors were used for the experiments. The metabolic activity of both microbiota over the incubation time was confirmed by the determination of SCFA levels in control incubations with and without DMSO (Fig. S16). All the alkaloids were stable over 24 h in abiotic incubations with microbe-free PolyFermS effluent with 30% nutritive medium buffered at pH 6.5. No major differences were observed between abiotic and microbiota incubations, suggesting that no microbiota-mediated metabolism of studied alkaloids occurred (Fig. 3).

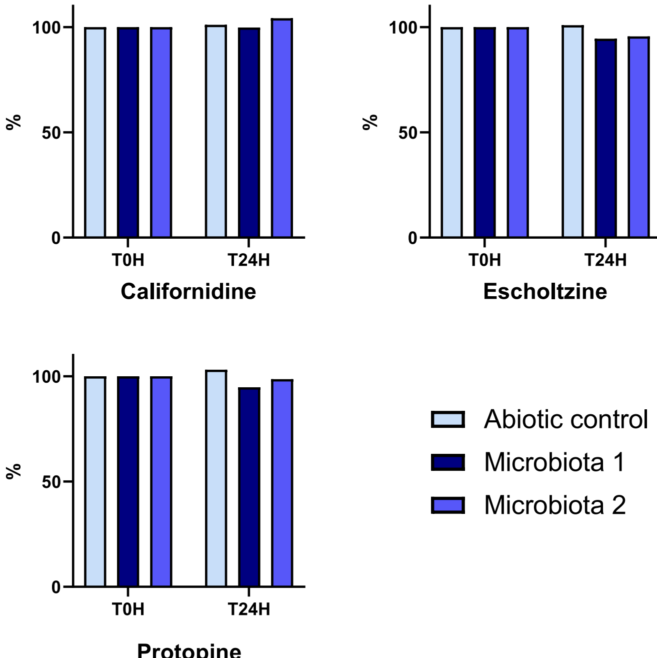

Fig. 3. Stability of compounds after 24 h incubation in PolyFermS effluent without active microbiota (abiotic), and in viable PolyFermS microbiota of donors 1 and 2 supplemented with 30% nutritive medium. All incubations contained 20 μL DMSO. Mean concentration values (n = 2) are normalized to 100%. The individual values of the two replicates are given in Table S25.

Upon ingestion of herbal medicines, phytochemicals are transported through the gastrointestinal tract and can be absorbed in the small intestine. The permeability experiments with the Caco-2 cell model indicated that californidine is low-to-moderately permeable (Table 2). Thus, relevant concentrations could possibly be reached in the colon. Moreover, our results with two different gut microbiota suggest that biotransformation of californidine in the colon is not to be expected. In contrast, escholtzine and protopine were found to be highly permeable in the Caco-2 cell model, which in turn would imply only low concentrations in the colon (Table 2). However, if a small fraction of these two alkaloids would not be absorbed in the small intestine, the results from microbiota incubation experiments suggest that they also would not be metabolized by the gut microbiota. While the disposition of escholtzine has not been investigated, protopine was found to have an absolute bioavailability of 25.8% in rats [29] and to undergo phase I/II metabolism [27]. In addition, excretion studies in rats have shown that

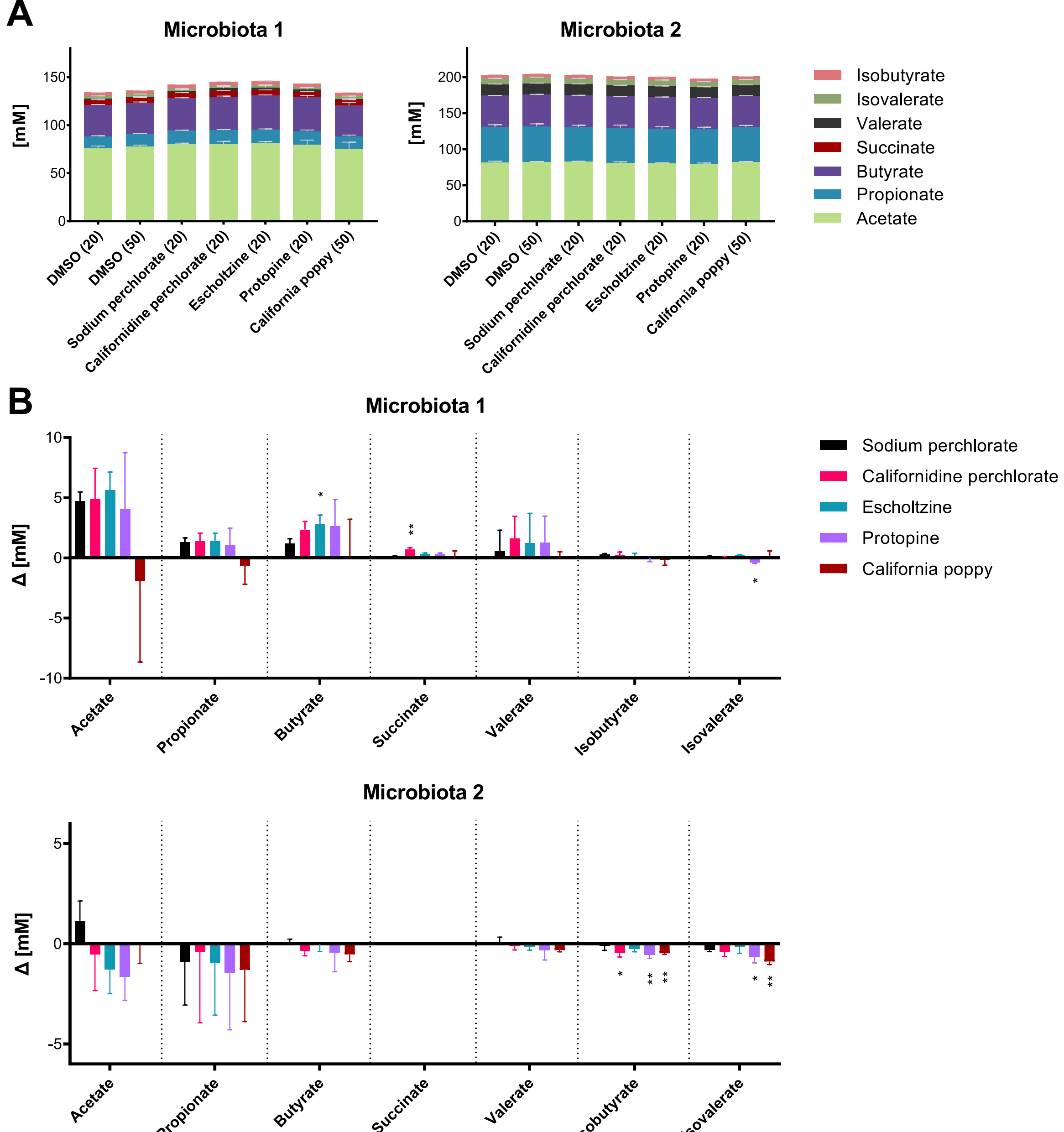

Fig. 4. (A) Fermentation metabolite concentrations after 24 h incubation of PolyFermS microbiota of donors 1 and 2, respectively. Incubations were supplemented with 30% nutritive medium and DMSO, with or without herbal compounds or extracts. Averages +/- SD. (n = 3). (B) Difference (Δ) after 24 h of short-chain fatty acid concentrations in compound vs DMSO control fermentation. Averages +/- SD. (n = 3). Asterisks indicate significant differences in average metabolite concentration between compound fermentation and respective DMSO control, analyzed by one-way ANOVA.

protopine was mainly excreted in metabolized form (>99%) [29]. Therefore, metabolites of protopine excreted via the hepatobiliary system could possibly transit over the gastrointestinal tract and interact with the gut microbiota. In the absence of data, this can also not be excluded for escholtzine.

3.4. Impact of compounds and plant extracts on microbiota activity and viability

Next, the impact of the California poppy extract and its compounds on the SCFA production by the two artificial gut microbiota was assessed. The main SCFAs (acetate, propionate, butyrate) and other minor organic acids (succinate, valerate, isovalerate, isobutyrate) were quantified and used as readout for the carbohydrate fermentation activity, which is a key metabolic function of the human gut microbiome [7]. The artificial microbiota derived from the two female fecal donors were of two distinct but prevalent microbiota types. Microbiota 1 was producing more butyrate (butyrogenic), while microbiota 2 was producing more propionate (propiogenic) (Fig. 4A). Californidine, escholtzine, protopine and the California poppy extract did not markedly impact the total SCFA levels (Fig. 4A). In microbiota 1, butyrate production increased by 9% in the presence of escholtzine, succinate production by 13% in presence of californidine, and isovalerate production decreased by 14% in the presence of protopine, while the effect of California poppy extract on individual SFCAs was negligible (Fig. 4B). In microbiota 2, isobutyrate levels decreased in the presence of californidine (−10%), protopine (−11%), and California poppy extract (−9%), while levels of isovalerate decreased in the presence of protopine

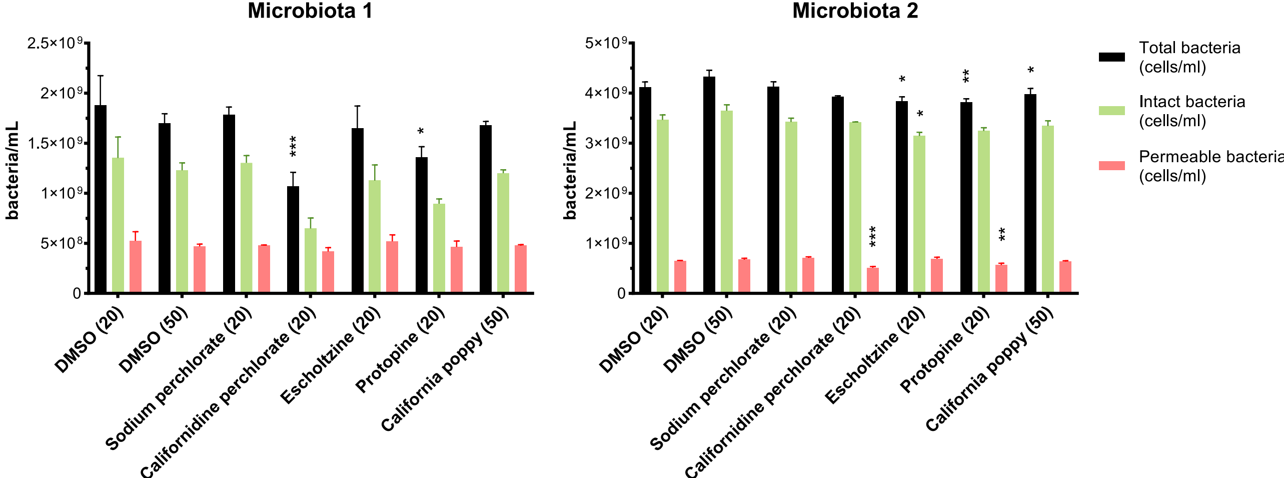

Fig. 5. Concentration of total (black), viable (intact, green) and dead (permeable, red) bacteria after 24 h incubation in PolyFermS microbiota of donor microbiota 1 and 2. Microbiota were supplemented with 30% nutritive medium and DMSO, with or without single compounds or herbal extract. Averages on bacteria/mL +/- SD (n = 3). Asterisks indicate significant differences in average bacterial concentration between compound fermentation and respective DMSO control fermentation analyzed by one-way ANOVA.

(−8%) and California poppy extract (−10%) (Fig. 4B).

Additionally, the impact of californidine, escholtzine, protopine and California poppy extract on bacterial concentration and viability was assessed by flow cytometry, whereby the total concentration of viable and dead bacteria in the microbiota after 24 h of incubation was determined. In microbiota 1, the alkaloids californidine and protopine resulted in significantly lower total bacteria concentrations (43% and 28% lower, respectively) compared to the control, which may be explained by a lower fraction of viable cells (trend, not significant) (Fig. 5). In microbiota 2, a small antimicrobial effect was noticed for californidine (with a decrease of 21% in permeable cells), escholtzine (with a decrease of 7% total bacteria and 9% in intact cells), protopine (with a decrease of 7% total bacteria and 13% in permeable cells) and the California poppy extract (with a decrease of 8% total bacteria) (Fig. 5).

Our data suggest that exposure to californidine, escholtzine, protopine and California poppy extract does not markedly affect the microbial fermentation activity and bacterial viability at the tested concentrations (30 μg/mL for compounds, and 500 μg/mL for the extract). However, it cannot be excluded that with higher test concentrations in the batch fermentation assays and with a long-term exposure in continuous fermentation experiments the SCFA metabolism and bacterial viability could be affected.

The quantitative analysis of the major alkaloids in commercial preparations of California poppy, together with manufacturer's dosage recommendations, showed that the maximum ingested amounts of californidine, escholtzine and protopine are in the range of 0.16–2.97, 0.10–1.11, and 0.02–0.31 mg/day, respectively (Fig. 2). Considering these amounts, an average volume of 200 mL of the proximal colon [30], and a colonic retention time of 8 h resulting in 600 mL proximal colon suspension per day [18,31], and assuming stability and no absorption of compounds in the upper parts of the gastrointestinal tract, the calculated concentrations of californidine, escholtzine and protopine in the colon would be in the range of 0.3–4.9, 0.2–1.8, and 0.03–0.52 μg/mL, respectively. Assuming a homogenous dispersion of compounds in the proximal colon, the concentrations used in our experiments were above the expected maximum theoretical colonic concentrations (about 6-fold for californidine, 17-fold for escholtzine, and 58-fold for protopine). Thus, one can reasonably assume that upon oral intake of California poppy herbal products the alkaloids would not substantially impair the fermentation capacities and the bacterial viability of microbiota. As to the California poppy extract, concentrations used in our experiments were up to 4-fold lower than those theoretically expected with a maximum recommended daily intake, where intestinal concentrations of 0.7–2.1 mg/mL could be reached. For the herbal products themselves an impact on microbiota balance cannot be excluded at this point. However, experiments with a higher number of individual PolyFermS microbiota would be needed to further substantiate the findings. Further, our experiments with high inoculation ratio were designed to assess the effect of the compounds on the microbial metabolite production and overall viability under limited growth [32]. Therefore, future in vitro continuous gut fermentations may allow to monitor long-term impact of alkaloids on microbial community structure and diversity.

4. Conclusions

The contents in californidine, escholtzine and protopine in eight commercial California poppy products were determined by UHPLC-MS/MS. In all products californidine was the major alkaloid, followed by escholtzine. The alkaloid content in the analyzed products varied significantly, ranging from 0.13 to 2.55 mg/g for californidine, 0.05–0.63 for escholtzine, and 0.008–0.200 mg/g for protopine. Based on the dosage recommended by manufacturers, maximal daily doses for the three alkaloids were calculated to be between 0.16 and 2.97, 0.10 and 1.11, and 0.02 and 0.31 mg/day, respectively.

Californidine was found to be low-to-moderately permeable, whereas escholtzine and protopine were highly permeable in the Caco-2 cell assay. The transport of each compound was possibly involving an active process. Escholtzine and protopine were also likely metabolized in Caco-2 cells.

The three alkaloids were not metabolized in the two PolyFermS artificial gut microbiota obtained from two different healthy female fecal donors. This suggests a negligible role of the gut microbiota in the disposition of the three alkaloids. The alkaloids and the extract did not markedly impact the SCFA production or the bacterial viability of microbiota. Thus, use of California poppy does not seem to affect gut microbiota metabolism, at least in short-term exposure. However, given the high interindividual variability of gut microbiota, studies with a larger number of microbiota and with continuous fermentations models are warranted to assess a possible effect on microbiota composition produced by a prolonged exposure.

With the calculated maximal daily intake in alkaloids, and assuming a 100% bioavailability, a rough estimation of the alkaloid concentrations in body fluids [33,34] for an average adult (40 years-old, 170 cm, and 70 kg) would theoretically result in maximal plasma concentrations

of 0.22, 0.08 and 0.02 μM for californidine, escholtzine and protopine, respectively. However, lower concentrations can be assumed for californidine due to its limited intestinal permeability. As for protopine, lower concentrations are also likely, given that intensive phase I and phase II metabolism has been shown in pharmacokinetic studies in rats [35]. Given the lowest IC_{50} values reported for these alkaloids in the study of Manda et al. (2016) [5] (IC_{50} of 100 μM for inhibition of any CYPs by californidine, IC_{50} of 0.3 μM for inhibition of CYP2C19 by escholtzine, and IC_{50} of 0.03 μM for inhibition of CYP2D6 by protopine), it seems unlikely that they could be responsible for herb-drug interactions.

Ethics approval and consent to participate

The Ethics Committee of ETH Zürich exempted this study from review because the sample collection procedure has not been performed under conditions of intervention. Informed written consent was obtained from fecal donors.

CRediT authorship contribution statement

Antoine Chauveau: Conceptualization, Methodology, Validation, Investigation, Writing – original draft. **Annelies Geirnaert**: Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Supervision. **Angela Babst**: Investigation. **Andrea Treyer**: Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Supervision. **Christophe Lacroix**: Conceptualization, Methodology, Validation, Supervision, Project administration, Funding acquisition. **Matthias Hamburger**: Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Olivier Potterat**: Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115420.

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